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INVOLVEMENT OF DOPAMINE IN THE NUCLEUS ACCUMBENS AND PREFRONTAL CORTEX IN COCAINE-ASSOCIATIVE LEARNING

Committee:

Christine L. Duvauchelle, Supervisor

Adriana A. Alcantara

R. Adron Harris

Rueben A. Gonzales

Timothy J. Schallert

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by

Aiko Ikegami, B.S.

Dissertation

Presented to the Faculty of the Graduate School of

the University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2003

INVOLVEMENT OF DOPAMINE IN THE NUCLEUS ACCUMBENS AND PREFRONTAL CORTEX IN COCAINE-ASSOCIATIVE LEARNING

Publication No. _____

Aiko Ikegami, Ph.D.
The University of Texas at Austin, 2003

Supervisor: Christine L. Duvauchelle

Stimuli formerly associated with cocaine-taking behavior are known to elicit physiological changes and craving in cocaine-dependent individuals. This is a result of learned associations between an environmental stimulus and the effects of cocaine, and is believed to be a major factor that leads to relapse in recovering cocaine addicts. A precise neural mechanism underlying how cocaine-paired stimuli produce craving and drug-taking behavior is currently unknown. Synaptic plasticity is known as a neural basis for associative learning. A modulatory role of a neurotransmitter, dopamine (DA), in synaptic plasticity has been implicated. Moreover, recent studies indicate that DA is particularly important during acquisition of associative learning, but less important as learning progresses. Yet, this notion has not been fully investigated using cocaine as a reinforcer. The nucleus accumbens (NAcc) and medial prefrontal cortex (mPFC) brain regions, are both largely implicated in drug addiction. Using an animal model of drug-

taking behavior in conjunction with an *in vivo* microdialysis technique, the dissertation experiments determined the involvement of DA in during distinctive stages of cocaine associative learning. Results from the experiments showed that NAcc DA was responsive to cocaine-paired stimuli during early, but not the late stages of cocaine associative learning while responsiveness of mPFC DA to cocaine-paired stimuli was enhanced with extended conditioning experience. The results indicate that brain areas responsive to conditioned stimuli transfers as associative learning progresses. These findings suggest that a dynamic role of DA in distinctive brain regions should be taken into account during treatment and relapse prevention of cocaine addiction.

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CHAPTER 1. GENERAL DESCRIPTION AND AIMS OF THE EXPERIMENT

Cocaine is a widely abused psychostimulant. The National Survey on Drug Use and Health in 2002 estimated that 33.9 million American ages 12 and older have experienced cocaine in their lifetime, while 2 million people are classified as current cocaine users. Cocaine is known to produce sympathomimetic activity, euphoria, a sense of well being and behavioral activation [198]. It is also known to produce a "jittery" and "unrelaxed" subjective feeling in some humans [115,290], and anxiogenic response in experimental animals [81,348]. In experimental animals, cocaine is reported to produce locomotor hyperactivity [14] and with high doses stereotyped behavior [198]. Cocaine-induced locomotor hyperactivity and stereotyped behaviors are known to be further augmented by repeated cocaine exposure [246]. This phenomenon is known as behavioral sensitization or reverse tolerance [198]. In humans, chronic cocaine intake is known to produce a paranoid psychosis that resembles a positive symptom of schizophrenia [39,244]. Reinforcing properties and abuse liability of cocaine was clearly suggested by early studies that observed experimental animals given unlimited drug access will easily learn to self-administer cocaine and will do so until their cocaine-induced death [37,79,345]. Thus, cocaine is a powerfully addictive reinforcing drug.

An earlier misconception about cocaine addiction was that withdrawal symptoms of cocaine were not severe since it did not produce gross physiological dependence compared to those produced by morphine or alcohol [198]. The addictive nature of cocaine, however, has been reconsidered, and Gawin in early 1990s advocated the

importance of psychological symptoms in cocaine withdrawal, including dysphoria, depression and intense craving, that may cause resumption of cocaine use [115]. In human cocaine addicts, relapse is often triggered by a painful emotional state, encounters with environmental stimuli associated with the drug use, and the drug itself [324]. Similarly, stress induced by foot shock or food deprivation, stimuli paired with cocaine, and a priming injection of cocaine have all been shown to reinstate cocaine self-administration behavior in experimental animals [3,97,142].

1-1. BASIC PHARMACOLOGY OF COCAINE

1-1-1. Metabolism

Cocaine is known to be readily absorbed by many routes [248], crosses the blood-brain barrier [198], and is rapidly distributed into the brain [230]. It is eliminated from the body with a plasma half-life of about 40 minutes [159]. Cocaine is biotransformed into its inactive major metabolites, benzoylecgonine and ecgonine methyl ester by liver and serum carboxylesterase, liver esterase and serum cholinesterase, and into an active minor metabolite, norcocaine, via hepatic mixed function oxdases [77,157]. Benzoylecgonine and ecgonine methyl ester are known as inactive metabolites [272]. Although norcocaine is self-administered by non-human primates [14], it was found to be formed only at negligible levels [35]. Thus, it suggests that it is the ability of cocaine itself, not its metabolites, to produce the reinforcing effects.

1-1-2. Plasma and brain cocaine levels and euphoric effect of cocaine

Peak plasma cocaine levels occur approximately at 15 to 60 minutes after intranasal application in humans. This is inconsistent with the maximum euphoria within 3 to 5 minutes reported by street users [312]. Thus, it was thought that peak plasma cocaine levels were not related with the maximum euphoric effect of cocaine. In the study however, cocaine was applied to surgical patients who were given the drug as a vasoconstrictor for nasal intubation and received various anesthetics prior to cocaine application, and apparently, subjective effects of cocaine were not measured. When plasma cocaine concentration and subjective effects were measured simultaneously in the same subjects, peak plasma cocaine concentration occurred within 5 minutes after intravenous administration and was highly correlated with the time course of maximum drug "high" experienced [158]. Therefore, the relationship between cocaine concentration and its euphoric effect was reconsidered.

An *in vivo* microdialysis study measured brain cocaine levels and took blood samples simultaneously from experimental animals after intravenous cocaine administration. Although the time course of the cocaine concentration in the brain and plasma closely paralleled each other, the peak cocaine levels in the brain appeared slightly later than that observed in the plasma levels [135,230]. However, longer sampling collection time for brain dialysates as compared to collection time for blood samples was employed, which may have resulted in the shift of the time course. When cocaine binding in human brain was measured by positron emission tomography (PET), the maximum binding occurred within 4 to 10 minutes after intravenous injection of radiolabeled cocaine [107] and the time course of the subjective effect of cocaine paralleled that reported by others [290].

Thus, it was suggested that the action of cocaine in the brain produces the reinforcing property of the drug.

1-1-3. Plasma and brain cocaine levels and sensitization

As it was mentioned, repeated cocaine exposure is known to cause augmentation of cocaine-induced locomotor hyperactivity in experimental animals [246], known as behavioral sensitization or reverse tolerance [198]. Mechanisms underlying the reverse tolerance to repeated cocaine administration is still controversial. However, Pettit and his associates showed that the brain cocaine concentration and plasma cocaine concentration in chronically treated animals were also augmented, suggesting increased cocaine bioavailability as a cause of behavioral sensitization [237]. In most of the sensitization studies, cocaine was administered either subcutaneously or intraperitoneally. However, when cocaine was administered intravenously, behavioral sensitization developed even though brain cocaine concentrations in chronically-treated animals were comparable with those receiving cocaine for the first time [228,229]. Thus, it is speculated that behavioral sensitization is independent of increased brain and plasma cocaine concentration observed after repeated peripheral administration of cocaine.

1-1-4. Monoamine transporters

Cocaine is known to block Na^+/Cl^- dependent plasma membrane transporters for monoamine neurotransmitters including dopamine (DA), serotonin (5-hydroxytryptamine; 5-HT) and norepinephrine (NE) [223]. Reuptake of the monoamine neurotransmitters via the plasma membrane transporters is known to be a primary means

of inactivation of released DA, 5-HT and NE in the brain [80]. Thus, cocaine causes increased synaptic or extrasynaptic DA, 5-HT and NE levels in the brain [252]. In rat brain homogenate, (-) cocaine, a naturally existing isomer of cocaine, has been reported to bind at SER transporter (SERT) with five fold more potency than DA transporter (DAT) and two fold less potency at NE transporter (NET) than DAT [255] while cocaine was found to be inactive at monoamine receptors [256]. The potency of (-) cocaine at cloned monoamine transporters from non-human primates was reported to be somewhat different from the potency found in the rats brain, and reported as rank order of DAT = SERT > NET [205]. At higher doses, cocaine is also reported to block M_2 muscarinic cholinergic receptors [284], sigma receptors [283] and voltage-dependent sodium channels [193]. Benzoylecgonine and ecgonine methyl ester are ineffective at the monoamine transporters and are not self-administered in animals [272], and norcocaine is effective at the monoamine transporters [255] and self-administered by animals [14]. This suggests that the reinforcing property of cocaine is mediated by monoamine transporter binding.

Among the monoamine transporters, the blockade of DAT has been strongly associated with the reinforcing effects of cocaine. Effective doses of cocaine and its congeners in self-administration studies were highly correlated with affinity for DAT while little relation was found with inhibition of either SERT or NET [256]. Moreover, monkeys did not maintain self-administration responses when cocaine was substituted with nisoxetine, a selective NET blocker [343]. When rats were pretreated with a selective DAT blocker, GBR 12909, alteration of their cocaine self-administration pattern was observed.

However, pretreatment with non-toxic doses of either selective NET inhibitor, desipramine and nisoxetine, or selective SERT inhibitor, fluoxetine, failed to alter the cocaine self-administration pattern [305]. A positron emission tomography (PET) study in humans revealed a significant correlation between self-report of cocaine "high" and DAT occupancy along with close temporal relationship of the "high" and the cocaine level in the brain [318]. These observations suggest the involvement of DAT inhibition in the brain mediates reinforcing effects of cocaine.

As mentioned above, cocaine causes increased synaptic or extrasynaptic DA, 5-HT and NE levels in the brain [252] by blocking the monoamine transporters [223] and thereby activating presynaptic and postsynaptic monoamine receptors. Since cocaine is reported to be inactive at the monoamine receptors [256], it is known as an indirect agonist of the monoamine transmission [198]. In this sense, monoamine receptor agonists or antagonists should influence effects produced by cocaine. Studies with receptor antagonists further support the relationship of DAT and the reinforcing properties of cocaine. For example, increased rate of cocaine self-administration was observed with pretreatment with low doses of DA receptor antagonists while high doses of the DA antagonists eliminated responding for cocaine in rats [100,148]. Similarly, DA receptor antagonist has been reported to shift dose response curve for cocaine self-administration while neither NE receptor antagonist nor 5-HT receptor antagonist altered cocaine self-administration patterns in monkeys [233,343]. These observations further implicated involvement of DA and DAT inhibition in reinforcing effects produced by cocaine.

1-2. NUCLEUS ACCUMBENS DOPAMINE

The main dopaminergic systems in mammalian CNS are composed of 1) the nigrostriatal pathway, originating in the substantia nigra and projecting to the neostriatum; 2) the mesolimbic pathway, originating in the ventral tegmental area (VTA) and projecting to brain regions, namely the nucleus accumbens (NAcc), hippocampus and amygdala; 3) the mesocortical pathway, originating in the VTA and projecting to the prefrontal cortex, limbic cortex, and hippocampus; and 4) the tuberoinfundibular pathway, originating in the hypothalamus and projecting to the pituitary gland [54,249]. Among these, the mesolimbic dopaminergic system has long been known as a neural substrate for the mediating reinforcing properties of appetitive stimuli [57]. Many types of appetitive stimuli such as food [139,337], stimuli associated with food [238], sex [72] and water for thirsty animals [354] are all known to increase DA levels in the NAcc. Drugs of abuse including cocaine, amphetamine, ethanol, opiates and nicotine are also reported to increase DA levels in the NAcc [84,139,349]. Moreover, destruction of the mesolimbic dopaminergic system at the NAcc with 6-hydroxy dopamine (6-OHDA) resulted in long-lasting decreases in responding for intravenous cocaine while lesions of NE projections did not [259]. Thus, the NAcc DA was thought to be a key neural substrate for reinforcing properties of cocaine [144,201,236,332]. Taken together, the blockade of DAT results in increased extracellular DA concentration in the NAcc, has been widely considered as a neural mechanism for the reinforcing properties of cocaine [183].

NAcc has been cytoarchitecturally and immunohistochemically divided into three subterritories: 1) The “core” which is the ventral and medial region of the NAcc, 2) the

"shell" which is lateral region of the NAcc, and 3) the "rostral pole" which is the rostral portion of the NAcc and lacks the "core" and "shell" boundary [20,321,357]. The NAcc "shell" is known to receive projection from the ventral prelimbic and infralimbic cortices [19], the midline and intralaminar thalamic nuclei, basolateral amygdala, rostromedial globus pallidus, ventral pallidum, subthalamic nucleus and the ventral mesencephalic cell groups that include A10 region [357] and projects to lateral hypothalamus, preoptic area, VTA, and medial ventral pallidum (VP), and it is also closely associated with extended amygdala [137]. The NAcc "core" is known to receive projections from widespread areas including the areas that send projections to the "shell" except the subthalamic nucleus and the globus pallidus [357], the anterior cingulate and prelimbic cortex [19], and projects to motor circuits such as substantia nigra, lateral hypothalamus, and dorsolateral VP [137]. Efferent and afferent projections of the "rostral pool" are known to possess both "core"-like and "shell"-like characteristics [357].

Both the "core" and "shell" of the NAcc receive dopaminergic projections from the VTA [357]. Are the afferent projections to the "core" and "shell" from the VTA the same population of DA neurons? Various studies suggest that distinct subpopulations of the VTA DA neurons may give rise to the dopaminergic projections to the "core" and "shell." For example, an *in vivo* microdialysis study showed that the "core" has higher DA concentrations than the "shell" [136]. Correspondingly, *in vivo* electrochemical recording in anesthetized rats showed that locally applied DA was more quickly removed from the extracellular space in the "shell" than in the "core" [74]. Contrary, electron micrographs showed that the "shell" has fewer DAT density [220], and greater synaptic

contacts and larger proportion of the tyrosine hydroxylase immunoreactive innervations as compared to the "core" [356], suggesting other mechanisms involving the rapid removal of DA in the "shell." Dense immunoreactivity of NE synthetic enzyme, DA β-hydroxylase, was found in the "shell" of the NAcc but not in the "core" of the NAcc [27]. DA has been reported to have a higher affinity for NET than DAT [255]. Thus, involvement of NET in the rapid removal of DA in the "shell" is speculated. Although it is yet to be determined, these observations suggest that afferent dopaminergic projections from the VTA to the "core" and "shell" may be composed of different neural populations.

Cocaine is also reported to have differing effects in the "core" and the "shell." For example, *in vivo* microdialysis studies showed that systemically administered cocaine profoundly increased extracellular DA levels in the "shell" but had only a slight effect on the "core" [136,243]. Correspondingly, *in vivo* electrochemical study showed that the clearance rate of cocaine-induced increases in DA levels substantially increased in the "shell" whereas the rate was unchanged in the "core" [74]. Less density of DAT density in the "shell" as compared to the "core" [220], and thereby greater "transporter saturation" and higher ratio of DA release to reuptake in the "shell" was speculated as a possible mechanism of sensitivity of this region to cocaine. If NET is involved in DA clearance in the "shell," it is odd to find the "transporter saturation." However, that may be caused by a lower affinity of cocaine to NET than DAT [255]. Finally, it was shown that ibotenic acid lesions of the sublenticular-extended amygdala, which is connected to the "shell," attenuated progressive ratio responding for intravenous cocaine while lesions of the subcommissural VP, which is connected to the "core," did not affect the responses

[262]. Thus, cocaine heterogeneously influences extracellular DA levels in the subterritories of the NAcc, and the "shell" may be more associated with the effects of cocaine than the "core."

1-3. COCAINE AND MONOAMINE TRANSPORTER KO MICE

The above mentioned pharmacological and behavioral observations, taken together, suggest that a blockade of DAT, which results in increased DA levels from the synapse at the NAcc, particularly the "shell" region, provide a neural mechanism for the reinforcing properties of cocaine. Recent findings from mice genetically lacking the DAT, however, have lead to reconsideration of the DAT hypothesis for the reinforcing effect of cocaine. Strikingly, mice lacking DAT were shown to still self-administer cocaine intravenously even in absence of putative sites for the reinforcing effects of cocaine [263]. Although there are possibilities that developmental adaptations occur in the lifelong absence of the gene products in the knockout mice, this finding suggests that DAT may not be exclusively necessary for producing rewarding effects of cocaine.

One of phenotypic characteristics reported in the DAT knockout mice is hyperactivity following placement in a novel environment. Homozygous DAT knockout (DAT $^{-/-}$ KO) mice are shown to have elevated locomotor hyperactivation and enhanced exploratory behavior in a novel environment [119,199,293]. On the other hand, homozygous NET knockout (NET $^{-/-}$ KO) mice were shown to rapidly habituate to a novel environment [199,344] while homozygous SERT knockout (SERT $^{-/-}$ KO) mice were indifferent from wild-type mice [292]. Interestingly, mice with double knockout of SERT and DAT genes

were shown to be even more hyperactive in a novel environment than mice with the deletion of DAT alone, yet knocking out the SERT gene has no effect on locomotor activity [292]. These studies suggest some synergistic influence of DAT and SERT on novelty-induced locomotor activation and that the novelty-induced locomotor hyperactivity is heavily affected by the absence of DAT.

As previously mentioned, cocaine is reported to induce locomotor hyperactivity in experimental animals [14], and cocaine-induced locomotor hyperactivity is further amplified by repeated cocaine exposure (e.g., behavioral sensitization) [246]. Likewise, intraperitoneal administration of cocaine was reported to produce locomotor hyperactivation in wild-type mice [119,293] as well as in SERT $^{-/-}$ KO mice [292], and induce behavioral sensitization in wild-type mice when administered repeatedly [344]. NET $^{-/-}$ KO mice were found to have greater cocaine-induced locomotor hyperactivity than wild-type mice, but failed to exhibit behavioral sensitization with repeated cocaine administration [344]. The already elevated locomotor activity of DAT $^{-/-}$ KO mice was shown to be unaffected by cocaine [119,293]. Although SERT $^{-/-}$ and DAT $^{-/-}$ double knockout mice were more hyperactive in a novel environment than the DAT $^{-/-}$ KO mice, the cocaine-induced locomotor hyperactivity in the double knockout mice and the DAT $^{-/-}$ KO mice were found to be comparable [292]. In these studies however, cocaine-induced locomotor hyperactivation or behavioral sensitization to cocaine might be masked by a ceiling effect of the novelty-induced hyperactivity some of these monoamine transporter knockout mice already possess. For instance, when knockout mice were given longer a

habituation period to an environment, and tested with intravenous cocaine, NET $^{-/-}$ KO mice showed a smaller magnitude of cocaine-induced locomotor hyperactivity as compared to wild-type mice and were indeed able to develop behavioral sensitization with repeated cocaine administration [199]. On the other hand, even with sufficient habituation to the environment, behavioral activity of the DAT $^{-/-}$ KO mice was shown to be unaffected by either acute or repeated cocaine administration [199]. Unfortunately, there was no comparable study conducted to test the cocaine effect on the locomotor behavior of SERT $^{-/-}$ KO and double knockout mice. Nevertheless, these observations suggest that the locomotor effects of cocaine are heavily dependent on the presence of DAT.

Drugs of abuse are known to produce a conditioned approach to places that have been associated with the drugs in the past, referred as conditioned place preference (CPP) [340]. The CPP paradigm is known to assess the ability of drugs to serve as positive reinforcers [200]. Cocaine is reported to produce CPP in rats [91]. Likewise, cocaine induces CPP in wild-type mice [292]. DAT $^{-/-}$ KO mice were also shown to exhibit intact cocaine-CPP [293]. Interestingly, NET $^{-/-}$ KO mice and SERT $^{-/-}$ KO mice were reported to show even greater preference to cocaine-paired compartment than wild-type mice [344]. The enhanced cocaine-CPP observed in NET $^{-/-}$ KO mice and SERT $^{-/-}$ KO mice was further augmented by combined deletion of both NET $^{-/-}$ and SERT $^{-/-}$ genes [133]. Contrary, even though SERT $^{-/-}$ KO mice have enhanced cocaine-CPP [344], DAT $^{-/-}$ combined with deletion of SERT abolished the cocaine-CPP [292]. Thus, it was

speculated that SERT inhibition by cocaine produces both reinforcing and aversive properties [292,310]. Although there is no study currently available to determine effects of double deletion of DAT and NET genes on cocaine-CPP, increased rewarding properties of cocaine observed in the NET $^{-/-}$ KO mice also leads to speculation of NET involvement in the aversive properties of cocaine [310].

In order to test the contribution of serotonergic and noradrenergic systems to cocaine reward and aversion, Hall and associates studied pharmacological blockade of SERT and NET in different types of transporter knockout mice [133]. In this study, SERT reuptake inhibitor, fluoxetine, which failed to produce CPP in wild-type mice and in SERT $^{-/-}$ KO mice, produced CPP in DAT $^{-/-}$ KO mice and NET $^{-/-}$ KO mice. The NET reuptake inhibitor, nisoxetine, on the other hand, failed to produce CPP in wild-type mice and NET $^{-/-}$ KO mice, but produced CPP in DAT $^{-/-}$ KO mice [133]. SERT and NET inhibitors did not induce CPP in wild-type mice yet they produced rewarding properties in the absence of DAT. This is suggestive of some neural adaptations occurring in the reward systems of these knockout mice [310]. Thus, the influence of serotonergic and noradrenergic systems on cocaine reward is still uncertain.

Blockade of DAT by cocaine and increased extracellular DA concentration in the NAcc has been widely considered as a neural mechanism for the reinforcing properties of the drug [183]. Yet DAT $^{-/-}$ KO mice were reported to self-administer cocaine [263] and exhibit intact cocaine-CPP [293]. Is cocaine able to increase the DA level in the NAcc of

the DAT $^{-/-}$ KO mice even in the absence of DAT? *In vivo* microdialysis studies showed that DAT $^{-/-}$ KO mice have unusually high concentrations of basal DA in the striatum that were about four to six fold higher than DA levels of normal mice [52,111,167,263]. Accordingly, an *in vivo* continuous amperometry study showed prolonged DA elimination rates and lower amplitude of DA release in the DAT $^{-/-}$ KO as compared to wild-type mice [18]. *In vivo* microdialysis studies found that the extracellular concentration of DA in the dorsal striatum (caudate putamen; CPu) of the DAT $^{-/-}$ KO mice was unaffected by cocaine [167,263]. On the other hand, cocaine was still able to increase DA levels in the ventral striatum (NAcc) [52]. Thus, it was suggested that cocaine is able to increase DA in the NAcc via blockade of other monoamine transporters [310].

Subsequent findings further support the involvement of NET in cocaine-induced increase in DA levels in the NAcc but not in the CPu in the DAT $^{-/-}$ KO mice. For example, GBR 12909, a specific DAT blocker, failed to increase DA levels in the NAcc of the DAT $^{-/-}$ KO mice. However, the NET blocker, reboxetine, which had no effects on the NAcc DA levels in wild-type mice, was able to increase DA levels in the NAcc of the DAT $^{-/-}$ KO mice [52]. Involvement of NET in the cocaine-induced increase in DA levels were further supported by the higher affinity of DA to NET as compared to DAT [255] and dense immunoreactivity of NE synthetic enzyme, DA β-hydroxylase, found in the "shell" of the NAcc but not in the CPu [27]. Thus, it was postulated that NET inhibition results in the cocaine-induced increase in DA levels in the NAcc in the absence of DAT [52].

In vitro voltammetry studies from brain slices showed that cocaine had no effects on either the rate of DA clearance or release in the striatum [167] and in both the "shell" and "core" of the NAcc in the DAT $^{-/-}$ KO mice, while the rate of clearance was prolonged in the wild-type mice [47]. The NET blocker, reboxetine, was reported to increase DA levels in the NAcc of DAT $^{-/-}$ KO mice in the aforementioned *in vivo* study [52]. Yet, the NET blocker desipramine, as well as the SERT blocker, fluoxetine, were unable to change the DA clearance in the NAcc brain slices of DAT $^{-/-}$ KO mice [47,167]. Thus, it was suggested that NET and SERT are not involved in actively clearing DA in the NAcc when there is no influence from the midbrain or other brain regions as result of deafferentation [47,167]. The *in vitro* findings led to a speculation that the cocaine-induced DA increase in the NAcc of DAT $^{-/-}$ KO mice is due to influences at cell body regions rather than alteration of clearance at the terminal region [47].

In summary, the observations from mice lacking DAT led to a speculation that blockade of DAT may not be exclusively necessary to produce rewarding effects of cocaine. For instance, DAT $^{-/-}$ KO mice still self-administer cocaine intravenously [263] and show intact CPP [293], though locomotor effects of cocaine were totally abolished by DAT deletion [119,199,293]. Cocaine was, however, still able to increase extracellular concentration of DA in the NAcc [52]. Thus, it was inferred that in the absence of DAT, DA can diffuse to neighboring non-dopaminergic cells where NET or SERT exist, and as a result cocaine increases DA levels in the NAcc [310]. *In vitro* voltammetry studies however, showed that cocaine had no effects on either the rate of DA clearance or release

in the NAcc, suggesting that NET and SERT is not directly involved in DA clearance at the NAcc [47,167]. The mechanisms underlying the cocaine-induced increase in DA levels of the DAT $^{-/-}$ KO mice are still unknown. The above mentioned knockout mice studies however, suggest that even though DAT may not solely elicit the reinforcing properties of cocaine, the involvement of DA in the NAcc cannot be refuted.

1-4. MIDBRAIN DOPAMINE NEURONS

As previously mentioned, the NAcc receives dopaminergic projections from midbrain VTA [249]. Midbrain DA neurons *in vivo* are reported to exhibit a tonic single spike with a long duration of a triphasic action potential (3-6 ms) or a bursting discharge of two to six pulses/sec [49,269]. In contrast to the activity observed *in vivo*, regular pacemaker activity was reported to the DA neurons *in vitro* [269,282]. Various stimuli are reported to activate the midbrain DA neurons. For example, single-unit recording of the DA neurons in freely moving rats showed that the DA neurons increased firing rate and burst activity in response to sensory stimuli and orienting responses [109] as well as to stimuli conditioned with rewards [206]. The DA neuronal activation is also observed with novel stimuli in monkeys [188] and salient stimuli in cats [147]. Similarly, a majority of midbrain DA neurons in monkeys are activated in response to food reward and stimuli conditioned with food reward. It was also noted that a larger number of neurons in the VTA and medial substantia nigra are activated compared to neurons in more lateral regions in the midbrain [273]. These observations suggest that the midbrain DA neurons *in vivo* are tonically active but respond to stimuli by burst discharges.

The activity of the midbrain DA neurons is also largely influenced by number of factors. For example, iontophoretic application of DA resulted in decreased firing *in vitro* [184,269]. Similar to the *in vitro* study, *in vivo* studies also showed that the DA neuronal firing decreased with systemic administration of a DA agonist, such as apomorphine, but increased with a DA antagonist, haloperidol, in anesthetized mice [269] and rats [48] as well as in non-anesthetized paralyzed rats [49] and freely behaving rats [109,206]. Iontophoretic application of DA directly into the brain region also resulted in similar changes in the firing rate *in vivo*; the effect also was blocked by systemic administration of haloperidol [269]. These observations from both *in vivo* and *in vitro* suggest that DA has an inhibitory effect on the midbrain DA neurons, with the effects possibly induced by impulse-modulating DA autoreceptors. Similarly, cocaine was reported to inhibit neural activity of the VTA neurons, and the inhibitory effects of cocaine are blocked by the DA antagonist, sulpiride, *in vitro* [50,184] and *in vivo* [94].

Activity of the midbrain DA neurons are reported to increase by iontophoretic application of glutamate, while N-methyl-D-aspartate (NMDA) receptor antagonists, (+-) 2-amino, 5-phosphonopentanoic acid (AP-5), or gamma-aminobutyric acid (GABA) reduce the occurrence of burst activity [61,126,206,269,282]. Morphine [194,307] and nicotine [240] are reported to excite the VTA DA neurons. Thus, these studies implicated the presence of GABA, NMDA, opioid, and nicotinic receptors that are capable of influencing activity of the midbrain DA neurons. It was noted however, that some of these drugs reduce burst frequency, but do not induce absolute inhibition, suggesting a modulatory role of the receptors [177].

1-5. "PHASIC" AND "TONIC" DOPAMINE RELEASE

When midbrain DA neurons are activated, it results in the release of DA from synaptic and asynaptic axonal varicosities at the terminal regions [56]. The DA neural transmission is reported to occur in two different ways, "phasic" DA release and "tonic" DA release [125]. Grace has proposed that the transient "phasic" DA release is induced by burst firing of DA neuron in response to behaviorally relevant stimuli that are largely influenced by dopaminergic, glutamergic, GABAergic, and cholinergic inputs [61,126,206,240,282]. On the other hand, "tonic" DA release is regulated by cortical glutamergic regulation and occurs in a spike-independent manner [125]. The "phasic" DA release is subjected to rapid removal from synaptic space by fast, low-affinity/high capacity re-uptake systems while the "tonic" DA release result in steady-state background levels of extracellular DA [125].

The "phasic" DA release was observed by an *in vivo* chronoamperometry study that showed that electrical stimulation of the VTA mimicking the bursting pattern resulted in "phasic" increase in extracellular DA levels in the NAcc. In this study, the released DA by a single pulse was readily eliminated between every action potential within less than 250 ms [62]. Since the "phasic" DA release is rapidly removed from the synaptic space [125] microdialysis measurements that lack high temporal resolution may not detect such "phasic" DA transmission induced by the burst firing [336]. However, a recent *in vivo* microdialysis study was able to show the "phasic" DA release by blocking the reuptake system. In the presence of a DAT inhibitor, burst neuronal activity of the VTA DA neurons resulted in an increase in DA levels that was not detected without the DAT

inhibitor. This increase turned out to be larger than a DA increase induced by increased population activity of the DA neurons detected in the absence of the DAT inhibitor [105]. Thus, it was suggested that a large DA release induced by the burst firing is indeed rapidly removed by reuptake, whereas increased population activity modulates "tonic" DA levels that are less influenced by the reuptake.

As suggested by Grace, the PFC is known to control "tonic" release of DA in the striatum [125,170]. Electrical stimulation of the PFC is reported to increase DA release in the NAcc [303]. Intra-medial PFC (mPFC) infusion of glutamate increased DA transmission in the NAcc and VTA neuronal activity [212]. Intra-mPFC infusion of DA antagonist has also been reported to influence basal DA levels in the NAcc, although the effect was only apparent 24 hours after the infusion [225]. Since DA in the mPFC is known to have a modulatory influence on the cortical glutamatergic projection to the NAcc and the VTA [178], involvement of glutamatergic influence on the NAcc DA levels is also inferred from the study. In addition to the PFC influence, "tonic" DA release is also reported to be influenced by glutamatergic projections from ventral subiculum (VS) of the hippocampus to the NAcc and VTA. For example, *in vivo* microdialysis studies showed that intra-VS NMDA injections resulted in increased firing rates of the VTA DA neurons and increased NAcc DA levels [45,185]. Whether prefrontal and hippocampal influences on the DA levels in the NAcc are mediated directly through the NAcc or indirectly via the VTA is still uncertain. Nevertheless, these observations clearly suggest cortical glutamatergic modulation of basal "tonic" DA release at the NAcc.

1-6. DOPAMINE RECEPTORS

The released DA that escaped DAT reuptake is known to exert its effects via postsynaptic and presynaptic DA receptors [56]. There are two general classes of DA receptors, termed D1-like and D2-like receptors. D1-like receptors include D1 and D5 types while D2-like receptors include D2, D3, and D4 types [326]. Synthesis-modulating, release-modulating, and impulse-modulating autoreceptors are known to belong to the D2-like receptors [80]. All of them are known to belong to a family of guanine-nucleotide-binding proteins (G-protein) coupled transmembrane receptors [249]. In the rat striatum, 80% of DA receptors are known to be D1-like receptors in which the majority of them are in low-affinity states and 20% of receptors are D2-like receptors that are primarily in high affinity states [254]. High affinity states are maintained in extracellular concentrations of DA that are achieved by the low steady "tonic" release. Therefore, Wightman and Robinson suggested that receptors with low affinity states are activated by a surge release of DA with "phasic" transmission, and thus high- and low-affinity states receptors are differentially activated by "tonic" versus "phasic" DA release [336]. If that is the case, then receptors activated by DA should be dependent on types of transmission (e.g., tonic or phasic), which are largely influenced by projections from other brain regions.

1-7. STRIATAL MEDIUM SPINY NEURONS

Major target neurons of the dopaminergic projections from the VTA are known to be GABAergic medium spiny neurons, which express both D1- and D2-like receptors and constitute more than 90 % of neurons in the striatum (for a review see [33]). Membrane

potentials of the medium spiny neurons are also known to be a determinant of action of DA on the neurons. Spontaneous activity of the striatal medium spiny neurons *in vivo* are reported to switch between two states, a hyperpolarized "down state" and a depolarized "up state" [338]. This is possibly due to the excitatory input from cortical area [125,170,178,212,303] and from hippocampus [45,185] since activity of the medium spiny neurons in the denervated striatal slice is reported to be in a quiescent "down" state [140]. When in the hyperpolarized "down" state, D1 agonist treatment is reported to have inhibitory effects on evoked discharge of the medium spiny neurons due to K^+ currents. When the neurons are in the depolarized "up" state, the D1 agonist potentiates the evoked discharge via PKA-cAMP-dependent enhancement of L-type Ca^{2+} currents [140]. On the other hand, effects of D2-like receptor stimulation are known to be inhibitory at any membrane state (reviewed by [59]). In a study by Hernandez-Lopez and associates, the application of DA resulted in similar enhancing effects with a D1 agonist [140], agreeing with the concept that majority of striatal neurons are D1-like receptors [254]. Synergistic effects of D1-like and D2-like receptors on enhancement of firing rate of the medium spiny neurons are possibly mediated by $\beta\gamma$ subunit of the G-protein [146], further complicating the question of which factors affect the activity of medium spiny neurons. Nevertheless, because of the membrane potential-dependent effects of D1-like receptors on the activity of medium spiny neurons, it was suggested that DA in the striatal neurons enhance response to strong excitatory input while it reduces weak transient input. Thus the signal to noise ratio of information reaching the brain region is increased before behavioral outcomes are mediated via the pallido-cortical pathway [140].

1-8. DOPAMINE AND INTRACELLULAR CASCADES

In addition to the effects of DA on postsynaptic potentials of the medium spiny neurons, DA is also known to initiate intracellular signaling cascades via binding to its receptors. D1-like and D2-like receptors are known to have opposite effects on cellular signaling cascades. For example, stimulation of the D1-like receptor is known to activate adenylate cyclase, while stimulation of D2-like receptor inactivates the enzyme [301]. Accordingly, adenylyl cyclase activation is known to increase formation of adenosine 3',5'-monophosphate (cAMP), causing cAMP-dependent protein kinase (PKA) activation and resulting in phosphorylation of a transcription factor, the cAMP response element-binding protein (CREB) [11,195,288] and a DA- and cAMP-regulated phosphoprotein of M_r 32,000 (DARPP-32) [222,323]. The phosphorylated form of DARPP-32 is reported to be a potent inhibitor of protein phosphatase-1 (PP-1), that in turn is known to regulate activity of voltage-gated ion channels and various neurotransmitter receptors [130]. On the other hand, inactivation of adenylate cyclase is known to inhibit the signaling pathway [195,222]. In addition, D2-receptor stimulation was found to phosphorylate DARPP-32 at cyclin-dependent kinase 5 (Cdk5) site of threonine residue (Thr-75) instead of PKA site of Thr-34 residue, which turns the DARPP-32 into an inhibitor of PKA [221]. Thus DA, as well as other neurotransmitters that have effects on the PKC signaling, is believed to regulate the phosphorylation and dephosphorylation of CREB and DARPP-32.

1-8-1. Effects of cocaine

Although cocaine is rapidly cleared from the brain [318] it is 10 times more potent than

DA at DAT [255] and increases DA levels in the extracellular space [252]. Thus, it is surprising to find out that only few studies actually showed the effects of cocaine on the signaling cascades [30,221,306]. This may be due to the fact that increased synaptic DA induced by cocaine is rapidly removed before it can affect the receptors that activate the signaling cascades, or that simultaneous stimulation of both D1-like and D2-like receptors cancel each other's effect. Another reason may be due to technical difficulties preventing the capture of phosphorylated and dephosphorylated transient states of the proteins. Whatever the reason, the existing studies showed that acute intraperitoneal injection of cocaine increased phosphorylation of DARPP-32 at the Thr-34 PKA site but reduced phosphorylation at the Thr-75 Cdk5 site in mice neostriatum [221]. On the other hand, chronic cocaine administration in rats was reported to increase phosphorylation of DARPP-32 at the Thr-75 site in the NAcc and CPu [30] and increase activity of adenylate cyclase and PKC in the NAcc [306]. Thus, acute cocaine might upregulate the signaling pathway while chronic cocaine downregulate the pathway.

1-8-2. DA, CREB, DARPP-32 and learning

CREB- and DARPP-32-signaling pathways are largely implicated in synaptic plasticity, learning and memory [1,51,110,130,151,195,217,288]. It was shown that DARPP-32 knockout mice are unable to form synaptic plasticity (both long-term potentiation, LTP, and long-term depression, LTD) in corticostriatal neurons [51]. DARPP-32 knockout mice are also reported to have deficits in reversal learning of a discriminative operant task for food reinforcement [141]. The importance of CREB in long-term memory and learning has been also observed by alteration of CREB genes in gill- and siphon-

withdrawal in *aplysia* [73], olfactory learning in *drosophila* [350] and fear conditioning and water maze in mice [36] and in rats [132]. Most of the learning deficits observed in these animals are apparent 24 hrs, but not immediately, after training, suggesting deficits in long-term memory while sparing short-term memory. Because D1-like and D2-like receptor stimulation have opposite effects on the CREB- and DARPP-32-signaling pathway, opposite effects of these receptors on learning are inferred.

By reviewing a significant amount of literature concerning effects of D1 and D2 receptor agents on appetitive learning of animals in operant and CPP paradigms, Beninger and Miller (1998) concluded critical roles for D1-like receptor stimulation in appetitive learning and D2-like receptor stimulation in the motor aspect of performance [17]. Recently, however, Eyny and Horvitz were able to disassociate the motor effects of D2-like receptor activation and demonstrated opposing roles of D1-like and D2-like receptors on the intracellular signaling cascades mirrored behavioral learning in animals. In the study, animals were trained to associate a tone with food under the influence of the D1 antagonist, SCH-23390, or the D2 antagonist, raclopride. Learning was assessed at a subsequent drug-free test session. They found that SCH-23390 disrupted, and raclopride facilitated appetitive learning in the animals [102]. However, the dose of SCH-23390 used in the study was within a dose range reported to produce conditioned place aversions in rats [287]. Thus, there is a possibility of learned associations between the tone and an aversive state produced by the drug that might in turn reduce the animals responses to the tone. Nevertheless, the above study suggests that both D1-like and D2-like receptors have modulatory roles in appetitive learning.

Involvement of DA in learning is further supported by *in vivo* electrophysiological studies in monkeys. As it was mentioned previously, midbrain DA neurons increase firing rate and burst activity in response to various sensory stimuli [109,147,188,206]. Using food reward, the response of the DA neurons becomes less prominent as the reward becomes fully predicted by repeated presentation [145,188,273,274]. Similar response patterns of the midbrain DA neurons have been reported when primary reward was conditioned with a neutral stimulus [188,207,251,273,275]. Whether activity of the DA neurons in response to stimuli other than appetitive rewards follows the same changes is currently unknown. However, these observations suggest that midbrain DA neuronal responses are dependent on learning stages. Since the DA neurons lose their responsiveness after learning is established, it was proposed that the activity of the midbrain DA neurons support and guide learning, particularly learning of stimuli that are appetitive in nature [17,273-275,334].

Taken together, the above findings on cellular mechanism, activity of the midbrain DA neurons, and animal behavior in learning paradigms suggest an essential role of DA on learning. Many types of drugs of abuse including cocaine are known to increase DA levels in the NAcc [84], and as a result the drugs recruit common molecular events that occur with learning. Learning and long-term memory formation is known to be associated with LTP and growth of new synaptic connections [169]. Single exposure of cocaine *in vivo* was shown to result in enhancement of the corticostriatal inputs to the VTA, similar to that associated with synaptic plasticity in hippocampus [311]. In the hippocampal slice, LTP has been shown to be linked with growth of new spines [96,192].

Repeated administration of cocaine or amphetamine was both shown to increase number of dendritic branches and spine density in NAcc medium spiny neurons and PFC pyramidal cells [260,261]. Because of the similarities in cellular events evoked by learning and drugs of abuse, the current view of drug addiction has centered its attention on the learning aspect of addiction [216,217].

1-9. DRUG ADDICTION AND LEARNING

Currently, drug addiction is considered as an aberrant form of learning [82,83]. Why is it aberrant? Di Chiara suggested that activation of the mesolimbic DA system by drugs of abuse are very different from that induced by conventional stimuli. DA release induced by conventional stimuli becomes less prominent after repeated exposure to the stimuli. On the other hand, drugs of abuse are resistant to such adaptation, resulting in persistent or sometimes even augmented DA release after repeated drug use [82]. It is important to note, however, that augmentation of cocaine-induced increase in NAcc DA levels found in animals exposed to repeated cocaine by intraperitoneal or subcutaneous routes [4,168,237] is associated with increased brain cocaine concentration [237]. Context-dependent and context-independent psychomotor stimulant-induced behavioral sensitization occurs under differing circumstances [25]. When cocaine is administered intravenously, cocaine bioavailability in the brain is unaffected even with chronic treatment [228,229]. The augmentation of NAcc DA increase in chronically treated animals as compared to cocaine-naïve animals is primarily due to the context in which animals received cocaine previously, since chronically treated animals tested in a novel environment did not show such augmentation [90]. Nevertheless, the pharmacological

effect of cocaine on NAcc DA transmission is persistent if it is not augmented, and is not suppressed by previous exposure of the drug. Contrary to the persistent effect of cocaine, increased DA release in the NAcc induced by a food reward was suppressed by repeated exposure [12]. Moreover, as mentioned previously, single-unit recording of midbrain DA neurons in monkeys also showed firing patterns of DA neurons in response to food rewards was reduced by repeated presentation [145,188,273,274]. These observations suggest that activation of the mesolimbic dopaminergic system is deviant from that induced by conventional rewards, and thus drug addiction is considered as a DA-dependent associative learning disorder. Such aberrant stimulation of the mesolimbic dopaminergic transmission by drugs of abuse is thought to strengthen drug-stimulus reward association, causing impulsive and compulsive drug-taking habits [295].

1-9-1. Cocaine associative learning

As mentioned in the beginning of this chapter, relapse to cocaine use can be triggered by various stimuli including environmental stimuli associated with the drug use [324]. In laboratory setting, stimuli formerly associated with cocaine-taking behavior are reported to elicit physiological changes and craving in cocaine-dependent human subjects [88,93,174,213,224]. In experimental animals, cocaine-paired stimuli have been reported to increase locomotor activity (a behavioral characteristic elicited by the drug itself) [43,106] and reinstate drug-seeking behavior in animals [277]. This is the result of learned associations formed between the pharmacological actions of cocaine and environmental stimuli present during cocaine taking [88].

This type of associative learning is known to involve both classical conditioning (also known as Pavlovian conditioning) and instrumental conditioning (also known as operant conditioning) [57]. Classical conditioning was originally described by Pavlov in1927, and it was demonstrated that repeated presentation of a neutral stimulus, termed as "conditioned stimulus," with a stimulus that automatically elicits response, termed as "unconditioned stimulus," results in the "conditioned stimulus" to become capable of eliciting autonomic responses originally produced by the "unconditioned stimulus" [231]. While classical conditioning involves learning associations between two stimuli, instrumental conditioning is known to involve learning associations between an operant behavioral response, such as approach behavior or avoidance behavior with reinforcer or punisher, respectively [54]. Stimuli conditioned via Pavlovian learning are known to acquire some of the properties of a goal directed behavior as we see in conditioned place preference [258]. In this sense, Pavlovian conditioning and instrumental conditioning are part of a learning continuum, in that instrumental conditioning cannot be developed without formation of Pavlovian conditioning.

1-9-2. Neural basis of associative learning

Perhaps the oldest theory for a neural basis of associative learning is the "Hebb rule" [134]. The "Hebb rule" suggests that after several pairings of a strong stimulus that causes postsynaptic activation with a weak stimulus that doesn't cause postsynaptic activation, the weak stimulus is strengthened and becomes capable of triggering postsynaptic activation by itself [280]. This was later demonstrated in the hippocampal slice, when it was shown that increases in synaptic strength (called LTP) is produced by

either brief, high frequency trains of stimulus or pairings with strong stimuli [172].

A simple form of associative learning has often been studied using the rabbit nictitating membrane response and classical conditioning of a tone with an air puff applied to the cornea [124]. It was found that after a few trials of conditioning, hippocampal neurons developed increased activity in response to the tone which closely paralleled the learned response of the nictitating membrane. Control animals that received unpaired stimulus presentations showed no such hippocampal activity [24]. Moreover, LTP of a rabbit's hippocampus induced by high frequency stimulation before conditioning training was shown to enhance rates of two-tone discrimination conditioning learning in the animals [23]. Attenuation of the conditioned hippocampal activity induced by lesion resulted in retardation of acquisition of such behavioral learning [28]. These observations suggest that associative learning induces LTP in hippocampus, and the hippocampal LTP is essential for normal development of simple form of associative learning.

More complex forms of associative learning such as fear conditioning, using tones paired with electrical shock is also reported to induce hippocampal neuronal activity [279]. The functional role of the hippocampal neuronal activity on behavioral learning is, however, reported to be transient and temporally restricted. For example, it was found that hippocampal lesions made one day after the fear conditioning training disrupted animals learned response to the tone. Animals showed increased fear responses with increasing time between the conditioning training and the day of lesion made (7, 14 and 28 days) [175]. This suggests that hippocampal activity is necessary for memory consolidation but

not for expression of learned response once memory is consolidated. A similar temporally limited role of hippocampus has been observed with a lesion study in non-human primates [360] and in an amnesiac human patient with hippocampal damage [296]. Thus, involvement of other structures in associative learning after memory consolidation is suggested [360].

1-9-3. Ventral tegmental area, Nucleus accumbens, dopamine and cocaine-associative learning

Neuronal learning is not unique to the hippocampus. LTP is also known to take place in all excitatory synapses in the brain [149] including the VTA [34]. The VTA DA neurons are known to express NMDA receptors [282], which are known to be essential in synaptic plasticity [342]. The DA neurons in the midbrain were shown to exhibit NMDA receptor-dependent LTP [34]. In fact, synaptic plasticity at excitatory synapses on VTA DA neurons is implicated as a neural mechanism of psychomotor stimulant-induced behavioral sensitization [271,342]. It is, however, important to note that an acute effect of cocaine in the VTA DA neurons is inhibitory [94], thereby it is odd if it causes LPT in the VTA DA neurons. Alternatively, neural plasticity induced postsynaptically at the NAcc medium spiny neurons was suggested as a mechanism for the behavioral sensitization [25]. Can neutral sensory stimuli, which may not be strong enough for "phasic" DA release at the NAcc, be strengthened via conditioning with cocaine to become capable of triggering "phasic" DA release and ultimately drug-seeking behavior? If such "Hebbian learning" occurs at the VTA DA neurons, then cocaine-paired stimuli may induce "phasic" DA transmission in the NAcc. On the other hand, if the "Hebbian

learning" occurs postsynaptically at the NAcc medium spiny neurons, then increased DA transmission at the NAcc may not be necessary to induce behavioral response to cocaine-paired stimuli.

Priming injections of cocaine are known to induce reinstatement of cocaine-seeking behavior [76]. The following observations led to a hypothesis that cocaine-seeking behavior elicited by priming injection of cocaine in animals are induced by increased DA transmission in the NAcc [300]. Elevated DA transmission in the NAcc evoked by either electrical stimulation of the VTA [239] and intra-VTA application of morphine [300] are reported to induce cocaine-seeking behavior in animals. Certain aspects of cocaine-seeking behavior are followed by repeated exposure to cocaine-paired stimuli are similar to those elicited by the priming injection of cocaine. Therefore, it was thought that drug-seeking behavior induced by cocaine-paired stimuli is mediated by the same neural system [234,257,299,333].

If increased DA transmission is the mechanism underlying both drug-seeking behavior induced by cocaine-paired stimuli and those induced by a priming injection of cocaine, DA agonists and antagonists should enhance or suppress drug-seeking behavior under those circumstances. Cocaine-seeking behavior induced by a priming injection of cocaine in conjunction with presentation of cocaine-paired stimuli was, indeed, attenuated by a non-selective DA antagonist [173]. The effects of D1-like and D2-like dopaminergic agents on cocaine-seeking behavior are not, however straightforward. For instance, reinstatement of cocaine-seeking behavior induced by a priming injection of

cocaine was enhanced by systemic administration of D2-like receptor agonist, 7-hydroxy-*N*,*N*-di-n-propyl-2-aminotetralin (7-OH DPAT), while D1-like agonists, SKF-82958 and SKF-81297, suppressed the priming effect of cocaine [281]. Another D2-like agonist, bromocriptine, was shown to have no effect on the cocaine-induced craving in cocaine addicts [156] while it enhanced reinstatement in animals [341]. Contrary, cocaine-seeking behavior induced by cocaine-paired stimuli in rats was attenuated by selective D3-receptor partial agonist, BP 897 [241], selective D1 antagonist, SCH-23390 [7,65,70] and SCH-39166 [65], D1 agonist, SKF-81297 [7], and D2-like antagonist, raclopride [70]. Bromocriptine has also reported to suppress craving induced by cocaine-paired stimuli in cocaine addicts [71]. While motoric or aversive effects produced by some of the agents may have confounded results of the aforementioned studies, why both agonists and antagonists produced similar effects on cocaine-seeking behavior is unknown. Nevertheless, it appears that effects of D1-like receptor stimulation are always inhibitory regardless to cocaine-seeking behavior induced both by cocaine-paired stimuli and by priming injections of cocaine. On the other hand, D2-like receptor stimulation is inhibitory on cocaine-seeking behavior induced by cocaine-paired stimuli, but enhances cocaine-seeking behavior induced by cocaine priming. Thus, a neural base of drug-seeking behavior induced by cocaine-paired stimuli might not be the same as behaviors induced by priming injections of cocaine. Thus, the involvement of NAcc DA in that aspect cannot be elucidated from the studies.

Because of opposite effects of D1-like and D2-like receptor stimulation in intracellular cascade [301] and behavioral learning [102], confounding effects of such dopaminergic

agents are expected. Thus, it is important to assess NAcc DA turnover in response to cocaine-paired stimuli. Many researchers in fact have attempted to elucidate the effects of cocaine-paired stimuli on NAcc DA transmission. However, results from various studies are not in agreement. For example, cocaine-associated stimuli are reported to induce increase in extracellular DA levels in the NAcc [90,91,106,117,179] and increase NAcc neuronal activities [108,214] while others find that responses of the NAcc is unnecessary to elicit conditioned response to cocaine-paired stimuli [42,43,65,215].

Such discrepancies in findings from the above-mentioned studies may be due to testing at different stages of associative learning. As mentioned previously, responses of midbrain DA neurons to primary rewards as well as to stimuli conditioned with the primary reward become less prominent as the reward becomes fully predictive through learned associations [145,188,207,251,273-275]. This suggests that midbrain DA neuronal responses are dependent on certain stages of learning. Are such dynamics of DA neuronal activity applied to stimuli conditioned with cocaine? Yet, drugs of abuse are known to powerfully and persistently stimulate the mesolimbic dopaminergic system, thereby suggested to strengthen stimuli-drug association [25]. Currently, there is no comparative *in vivo* single unit recording or microdialysis study available that has determined the learning-stage dependent effects of cocaine-paired stimuli on the midbrain DA neuronal activity and DA turnover, respectively.

In answer to the question, the following experiments were conducted. *In vivo* microdialysis was performed to determine the influence of cocaine-paired stimuli on DA

transmission at two dopaminergic terminal regions of the midbrain VTA neurons, the NAcc and prefrontal cortex (PFC), both of which are largely implicated in cocaine addiction, during an early and late stage of cocaine-associative learning. This is the first study that has determined the involvement of the mesolimbic and mesocortical dopaminergic systems in the conditioned effects of cocaine across different stages of cocaine-associative learning.

1-10. AIMS OF THE EXPERIMENTS

Experiment 1: To determine distinctive stages of cocaine-associative learning from animals cocaine self-administration pattern and behavioral reactivity to cocaine-paired stimuli during conditioning training sessions. This was achieved by monitoring the lever-response pattern and cue-reactivity of animals during discriminative cue conditioning self-administration sessions. Results from the experiment determined when to measure dopaminergic and behavioral responses to cocaine-paired stimuli during distinctive stages of cocaine-associative learning (i.e., early and late phase).

Experiment 2: To determine if cocaine-paired stimuli induces changes in NAcc DA levels and behavioral activity during an early or late stage of cocaine-associative learning. This was accomplished using animals that underwent either the limited or protracted conditioning sessions. *In vivo* microdialysis was performed to measure NAcc DA levels from rats self-administering cocaine or saline in the presence of either cocaine- or saline-paired stimuli. Locomotor activity of the animals during the test session was also measured.

Experiment 3: To determine if PFC DA levels are influenced by cocaine-paired stimuli during an early or late stage of cocaine-associative learning. The same experimental procedure as Experiment 2 was employed, with the exception that dialysates were collected from the mPFC of animals.

CHAPTER 2. EXPERIMENT 1: COCAINE-TAKING PATTERN AND CUE REACTIVITY DURING COCAINE-ASSOCIATIVE TRAINING

The main purpose of Experiment 1 was to determine distinctive stages in cocaine-associative learning. This was achieved by monitoring the lever-response pattern and cue-reactivity of animals during discriminative cue conditioning intravenous self-administration sessions (details in Methods). The following introduction section contains a brief discussion of why intravenous self-administration is a suitable paradigm for testing cocaine-associative learning.

2-1. INTRODUCTION

Drug self-administration models require animals to engage in earning of drugs by performing instrumental behavior [127]. Thus, the self-administration method is thought to model human drug-taking behavior. In early addiction research, drug self-administration in experimental animals was restricted to oral self-administration. The voluntary oral intake of cocaine was particularly not successful due to the possible aversive bitter taste of cocaine [186,304]. Sufficient volumes of voluntary drug intake was only achieved by manipulations, such as restricting food intake [103,304] or masking the initially aversive taste by sucrose [268]. Permanent intravenous jugular vein catheterization with head attachment apparatus, introduced by Steffens in late 1960s [297] and modified by others [218] in conjunction with the fact that experimental animals easily self-administer psychomotor stimulants intravenously [163], made it possible for

experimental animals to self-administer cocaine without such manipulations and obviously contributed to expand our knowledge about cocaine addiction.

It has been reported that animals easily acquire a lever-pressing response to obtain a secondary reinforcer [298]; a stimulus previously associated with a primary reinforcer. This suggests that the stimulus itself is capable of gaining conditioned reinforcing properties. Similarly, Everitt and his associates demonstrated that animals can learn a new instrumental response to gain access to a stimulus associated with intravenous cocaine. In the study, however, when cocaine was administered by a 'yoking' procedure, in which drug administration was not contingent on animals' behavior, the yoked animals failed to acquire the response. Thus, it was suggested that anxiogenic effects outweigh the rewarding property of cocaine when animals had no control over their intravenous infusion of cocaine [101]. Additionally, the dopaminergic response to cocaine was shown to differ between rats that received cocaine in such 'yoking' procedure and those self-administering cocaine [138,179]. An anxiogenic stress-like response to cocaine such as suppressed locomotor activity [81] along with increased plasma stress hormones such as corticosterone [348] is often observed with intraperitoneally administered cocaine. The elevated corticosterone levels induced by stress have been reported to influence learning and memory [209,265], thereby possibly influencing the results of experiments. These observations suggest the importance of the self-administration procedure.

Using an intravenous self-administration paradigm combined with discriminative cue conditioning training, the development of cocaine-taking behavior was monitored by

observing lever responses across self-administration sessions. The lever response patterns alone however, cannot determine the influence of conditioned stimuli, since once animals press the lever, the infused drug influences subsequent lever responses [76]. Thus, in conjunction with the lever response patterns, locomotor activity immediately after the presentation of conditioned stimuli but before the initial lever response ("cue reactivity") was also monitored. The cue reactivity measure provides additional indication of how animals develop behavioral responses to conditioned stimuli. The data from the lever response patterns and cue reactivity were used to differentiate two distinctive stages of cocaine-associative learning (i.e., an early stage and late stage).

2-2. MATERIALS AND METHODS

Subjects: Male albino Sprague-Dawley rats (Animal Resource Center, Austin, TX) weighing approximately 250 – 300 g at the beginning of the experiments were used (N = 39). In order to minimize the number of animals utilized, these animals were also used in the subsequent microdialysis studies as a prolonged conditioning group in Experiments 2 and 3. The animals were group-housed and maintained on a 12 hr. reversed light/dark cycle (light on 7:00 p.m. to 7:00 a.m.). In order to accustom animals to be handled and reduce discomfort that they may experience during the experiment, they were handled daily at least one week prior to the initiation of the experiment and continuously handled throughout experiments. Food and water were available *ad libitum* in the home cage except during food training.

Apparatus: Food training and self-administration sessions were conducted in identical one-lever operant chambers (28 x 22 x 21 cm) constructed of Plexiglas located in sound-attenuating chambers. Two sidewalls were constructed of metal with a single retractable operant lever on one side of the wall. A stimulus light was located above the retractable lever and a house light was located on the opposite side of the metal wall. Three sets of photocells were located on the front and back wall of the chamber, in the center and at 5 cm from each sidewall. The injector system was connected to a swivel mounted on a counterbalanced arm at the top of each chamber. One end of the swivel was connected, via polyethylene tubing (Tygon Microbore 1.5 mm o.d.), to a 10 ml syringe mounted on a syringe pump (Razel ®, Model A, 33.3 rpm). A spring-covered catheter (Plastics One) connected the other side of the swivel to the catheter termination mounted on the top of the animal's head. The experimental programs were controlled and data were collected by a Med Pentium 100 MHz computer using Med-PC software.

Food Training: In order to facilitate operant learning for food rewards, animals were food restricted (≈ 6g of standard rat chow per day) and trained to lever press for food on a Fixed Ratio 1 (FR1) schedule of reinforcement. Each lever response resulted in dispense of one sucrose pellet (45 mg, Noyes). After animals acquired the lever press for food, 10-min food reinforced operant sessions (FR1) were conducted for the next 6 days without food restriction.

Surgery: Animals were implanted with a chronic silastic intravenous jugular catheter (0.6 mm o.d.) under sodium pentobarbital (Nembutal, 50 mg/kg, i.p.) anesthesia.

Atropine sulfate (250 μg, s.c.) was given prophylactically to prevent respiratory tract secretions. Chloral hydrate (80 mg/kg, i.p.) was given, if necessary, to prolong the anesthesia. The free end of the catheter with a cannula termination (Plastics One) was passed subcutaneously on the side of the neck, out an incision in the animal's head and mounted on its skull. The catheter cannula was affixed to the skull with four stainless steel screws and dental acrylic cement. These animals underwent a minimum of one-week recovery prior to the beginning of the experiment. After the surgery, animals received 0.1 cc of saline solution consisting of one U/ml streptokinase, 67 mg/ml of the antibiotic, Timentin, and 30 U/ml heparin through their intravenous catheters everyday for a week. In order to keep the catheter patent, animals continued receiving the same solution without Timentin daily throughout the experiments.

Cocaine/Saline Conditioning Self-administration Sessions: Over the course of conditioning sessions animals had alternating days of cocaine and saline availability during one-hour daily self-administration sessions (i.e. 20 days of cocaine and 20 days of saline sessions). In the first 30 min of these sessions, the chamber is darkened and the lever is retracted for habituation to the chamber. After 30 min, the house light illuminates, the lever is presented, and cocaine or saline then becomes available for 30 min. During these sessions, each lever press resulted in the delivery of 0.5 mg/kg/0.1 cc cocaine hydrochloride mixed in isotonic saline (cocaine days), or saline alone (0.1 cc, saline days), infused over 6 seconds. The dose of cocaine (0.5 mg/kg) was chosen because it is in a range of intravenous cocaine commonly abused by humans, 0.35 mg/kg to 0.71 mg/kg [318] (25 to 50 mg [314]), and reported to be easily self-administered by

non-human primates [38] and rodents [236]. After each infusion, there was a 20-sec "time-out" period to prevent overdose from continuous infusion. During the "time-out," the lever was retracted, the stimulus light was off and no infusion was available. Cocaine-overdose was also prevented by programming computer to end a session when an animal reached the maximum lever pressing per session. The maximum lever pressing was set to 29 infusions, equivalent to a dose of 14.5 mg/kg/30 min, which is below the cocaine LD_{50} (17.5 mg/kg for an intravenous route in rats, The Merck Index, 1996). When the maximum lever pressing was reached, the lever was retracted and the animal was removed from the operant chamber.

Environmental Cues: Visual and olfactory environmental cues were introduced into the operant chamber immediately following the 30-min habituation periods. Visual cues consisted of either black or white felt "walls" attached to the sides of the clear Plexiglas operant chamber. Olfactory cues consisted of an oil-based scent (e.g., cinnamon or rose) saturated on a cotton ball located under the grid floor of the operant chamber.

Response Latency and Cue Reactivity Measure: Cue reactivity, defined as photobeam interruptions immediately after cue presentation and until initiation of the first lever response during the conditioning sessions, was measured over the course of 40 conditioning sessions. Due to a large individual differences, the cue reactivity counts from each day were combined into five days block and the mean for the five saline days was subtracted from that for the corresponding cocaine days. Response latency, i.e., time elapsed immediately after the cue presentation and before an animal made the first lever

response, was also measured throughout the 40 days of conditioning sessions. Since locomotor hyperactivity induced by cocaine-paired stimuli is short-lived and endures only about 100 sec (unpublished observations), the data was omitted from further analysis when animals didn't initiate the lever responses within 100 sec.

Data analysis: The number of lever pressing for cocaine and saline during the daily conditioning sessions was analyzed by a two-way analysis of variance (ANOVA, treatment x day) with repeated measures on days. When there were significant overall effects, *Post hoc* analyses (Fishers LSD) were used to detect significant differences at specific time points.

2-3. RESULTS

Lever responses during the self-administration training sessions: Fig. 1 shows number of lever responses during the conditioning sessions (N = 39). A two-way ANOVA (Treatment X Day) with repeated measures on the Day factor revealed no significant differences between Treatments (cocaine versus saline) [$F(1,76) = 0.7476$; NS], but significant Day [$F(19,1444) = 14.8761$; $p < 0.01$] and Treatment by Day interaction effects [$F(19,1444) = 3.8256$; $p < 0.01$]. *Post hoc* tests revealed that animals responded to cocaine significantly more at the late stage of training (day 7~20) than the early stage of training (day 1 ~ 4) whereas lever responses for saline at the early and late stages were compatible. *Post hoc* tests also showed that animals lever pressed significantly more during saline days than cocaine days during the early phase of training,

but started responding significantly more during cocaine days at the late stage of the training.

Cue reactivity during the self-administration training: Fig. 2-A shows the cue reactivity during the conditioning sessions (N = 7). A one-way ANOVA revealed a significance effect of Training stages [$F(3,18) = 3.5112$; $p < 0.05$]. The animals became more active after presentation of the cocaine-paired cues than that for saline at the late stage of training. *Post hoc* test revealed that the cue-reactivity was significantly different between the late (day 16-20) and early stages (day 1-5 and day 6-10).

Cue reactivity and lever responses during the self-administration training: When the lever responses were plotted as the same manner with the cue reactivity (Fig. 2-B), it revealed a positive correlation ($R^2 = 0.8677$). During the early stage of the training (day 1~10), when animals were more active following presentation of the saline-paired stimuli than the cocaine-paired stimuli, they self-administered more saline than cocaine. During the middle stage of the training (day 11~15), the animals were equally active following presentation of the cocaine-paired stimuli and saline-paired stimuli, and they self-administered equally as well. During the last stage of the training (day 16~20), the animals became more active following presentation of the cocaine-paired stimuli than the saline-paired stimuli, and self-administered significantly more cocaine than saline.

Response records during self-administration sessions: Fig. 3 illustrates characteristic response patterns for cocaine across different stages of self-administration sessions.

Accelerated lever responses for saline at the early training phase were observed, and responses for saline eventually ceased in the animal.

Response latency during the self-administration training: Fig. 4 shows the response latency from the animals (N = 39) during the cocaine self-administration sessions. A one-way ANOVA showed no significance effect of day [F (19,759) = 0.9461; N.S.].

Fig. 1

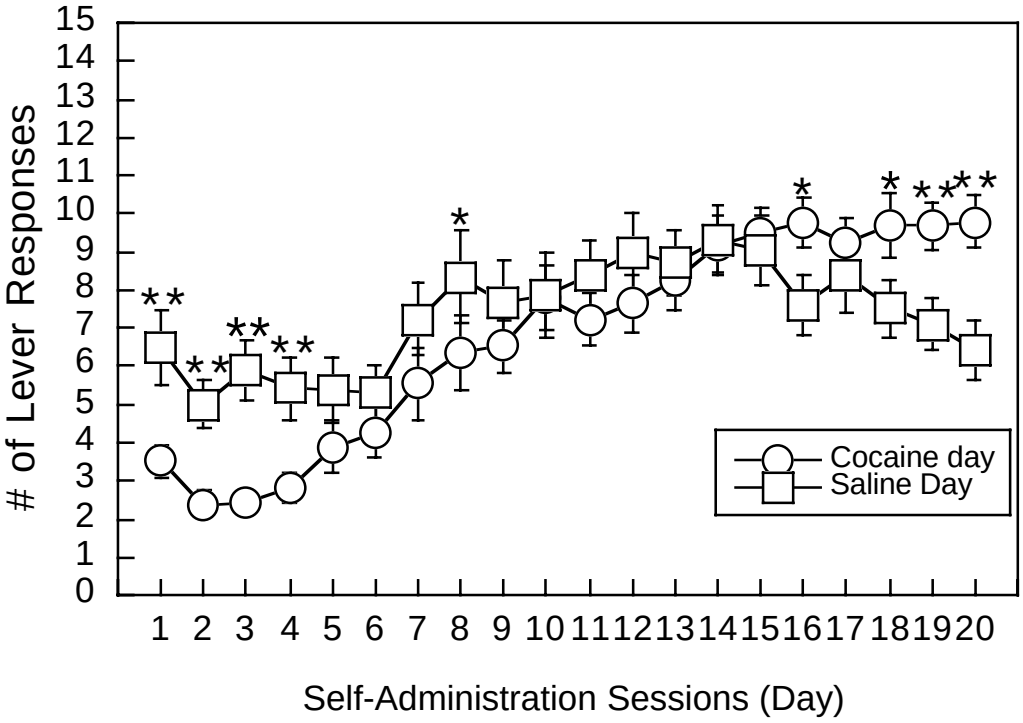

Fig. 1. Lever responses during the self-administration sessions for "cocaine days (○)" and "saline days (□)" (N = 39): Animals lever pressed significantly more during saline days than cocaine days during the early stage of the training, but lever pressed significantly more during cocaine days than saline days during the late stage of training (* and ** represent $p < 0.05$ and $p < 0.01$, respectively).

Fig. 2

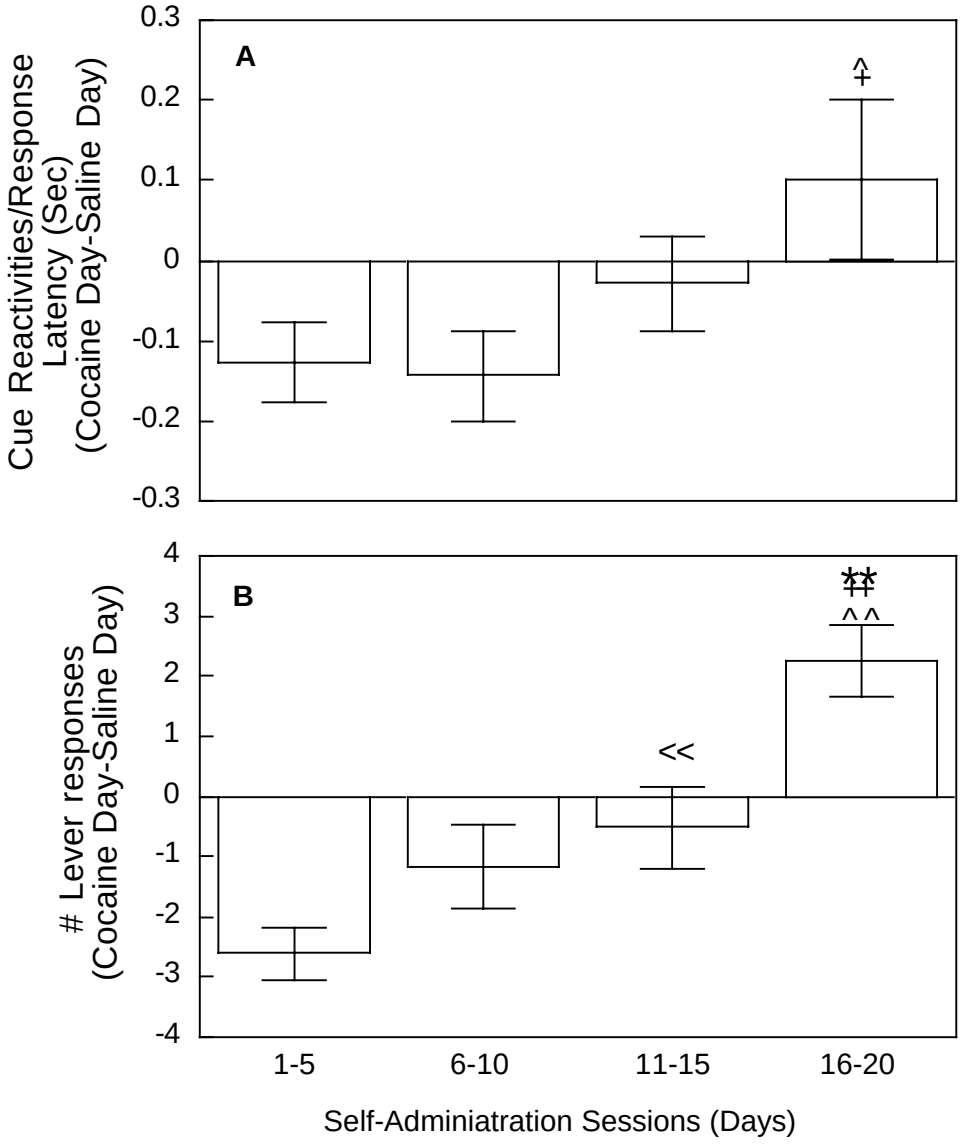

Fig. 2. (A). Cue reactivity of animals during the conditioning sessions (N = 7). The cue reactivity during day 16-20 was significantly different from those during day 1-5 (^, $p < 0.05$) and day 6-10 (+, $p < 0.05$). **(B)**. For better comparison, number of daily lever responses was plotted as the same way with the cue reactivity; each block represents mean lever presses for five days (N = 39). A one-way ANOVA revealed a significance effect of conditioning stages [$F(3,114) = 14.8601$; $p < 0.01$]. *Post hoc* test revealed the mean lever presses during the day 1-5 were significantly different from those during day 11-15 ($P < 0.01$, <<) and day 16-20 ($P < 0.01$, **). The lever presses during day 16-20 were also significantly different from those during day 6-10 ($P < 0.01$, ++) and during day 11-15 ($p < 0.01$, ^^).

Fig. 3

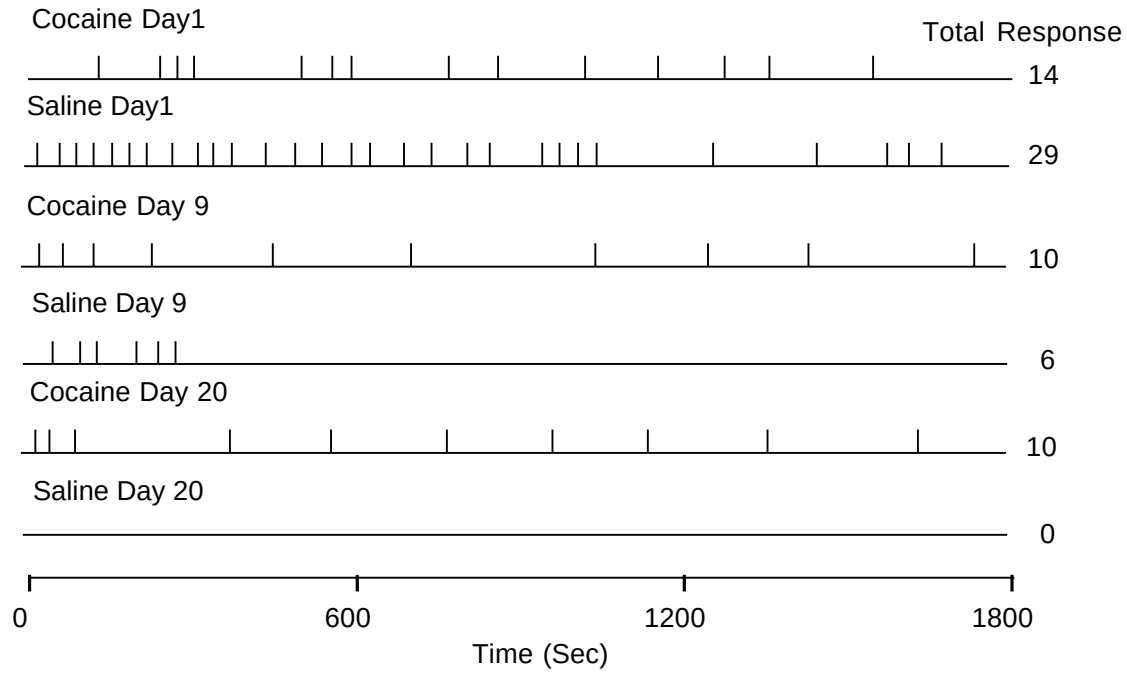

Fig. 3. Response records from a representative animal during the self-administration sessions across different stages of training. Vertical line indicates each lever press.

Fig. 4

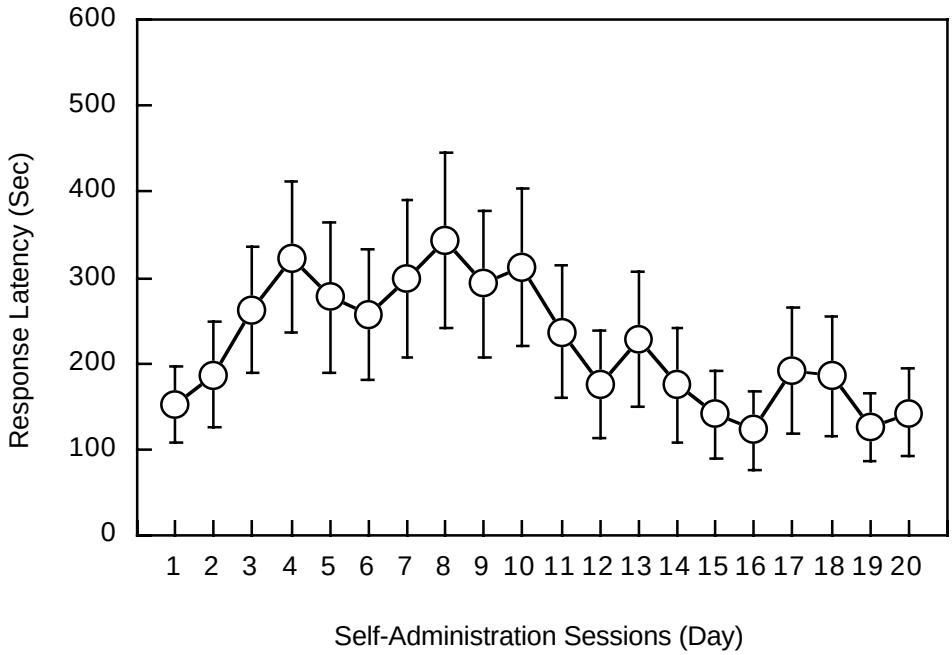

Fig. 4. The mean response latency from the animals (N = 39) during 0.5 mg/kg cocaine self-administration sessions. Although a one-way ANOVA failed to show a significance effect [F (19,759) = 0.9461; N.S.], possible due to a large individual variability, it shows that animals hesitate to initiate lever pressing for cocaine on days 3-10 while they progressively became faster at the late stage of self-administration sessions.

2-4. DISCUSSION

Results from the present experiment illustrate the animals' development of self-administration patterns during discriminative operant training. Robust lever responses for cocaine appeared after 7~8 days of cocaine self-administration sessions. The animals continued to increase their cocaine intake until the day 16, and maintained the level thereafter. The animals took quite a long time (i.e., 18 days each of cocaine- and saline-self-administration sessions) to reach a distinctive lever response pattern for cocaine and saline. This may be due to the relatively moderate dose of cocaine (0.5 mg/kg) used in the study, though this dose was reported to be easily self-administered by non-human primates [38] and rodents [236]. This dose is also in the range reported to be commonly abused by humans [318], and when self-administered by primates is reported to result in plasma cocaine levels [38] comparable to reported human euphoria [158]. Since most of our animals had acquired robust self-administration behavior after 7 ~ 8 days of self-administration sessions, the delay to exhibit distinctive lever responses for cocaine and saline was likely due to the training schedule utilized in the study. For instance, cocaine was only available every other day in the study, and for a limited duration (30 mins). We observed accelerated lever responses for saline at the early training phase, although responses for saline subsequently decreased and eventually ceased in some animals (Fig. 3). Bursts of responding after not receiving expected food reward or cocaine infusion is referred as "frustration responding," and commonly observed prior to cessation of lever responding in an "extinction" procedure [339]. For example, robust lever responses were reported when non-rewarding saline was substituted for cocaine [329] or amphetamine

[351]. Pretreatment with a preferential DA D2 receptor antagonist pimozide is also reported to induce a burst of "frustration responding" before extinction [351,352]. Thus, the animals in the present study may also have difficulty suppressing lever responses for saline during the early stage of self-administration training.

Lever responses of our animals show a tri-phasic pattern (Fig. 1). The animals started off responding more for saline than cocaine, then equally responded to cocaine and saline, and finally responded more for cocaine than saline at the late stage of the training. The tri-phasic self-administration pattern closely paralleled their cue reactivity (Fig. 2-A & B), suggesting that the cue reactivity is reflective of cocaine-seeking behaviors. Unlike the animals in the present study, it was reported that cocaine-taking behavior was uncorrelated with their cue reactivity in cocaine-dependent humans, [88,92]. In the studies with human subjects, however, cocaine was not available immediately after the cue exposure because of obvious ethical reasons. Thus, it was suggested that cocaine-paired stimuli exposure to human subjects in the laboratory setting is different from real world situation, because there is no real expectancy [92]. Nevertheless, similar correlations have been reported between cue reactivity and desire to drink [67], severity of craving cocaine [213] and cigarette smoking in smokers who are not attempting to quit [87]. Thus, cue-reactivity could be a good predictor for subsequent cocaine-taking behavior in laboratory animals.

It is note-worthy that a low level of activity was observed following cocaine-paired stimuli at the initial stage of training in the animals. This might be due to a

stress/anxiety-induced freezing behavior related to cocaine. Anxiogenic properties of cocaine have been reported in some humans [115]. In laboratory setting, cocaine-experienced humans reported a "jittery" and "unrelaxed" subjective feelings at the same they experienced "rush" and "high" following intravenous cocaine administration [290]. Similarly, cocaine has been reported to have "conflicting" properties, thus possessing both reinforcing and aversive properties, and resulting in approach and avoidance behaviors in experimental animals [99]. For example, when animals were trained to traverse a runway for intravenous cocaine delivered in the goal box, they exhibited "stop and retreat" behavior defined as a stop in front of the goal box and back toward the start box [99]. This behavior was similar to that found when food reward was simultaneously given with foot shock [116]. Such "stop and retreat" behavior is reduced dose-dependently by pretreatment with diazepam [99]. Interestingly, it was found that cocaine produced the anxiogenic effect on non-anxious animals while already anxious animals were not affected by cocaine [264], suggesting individual differences for such anxiogenic action of cocaine. Some of our animals also showed hesitation to initiate lever pressing for cocaine during the early stages of training, similar to the "stop and retreat" behavior. For example, our animals often approached the lever and put their paws to the lever without pressing it. This type of behavior might have increased the response latency for cocaine self-administration in our animals. The response latency for cocaine, however, was reduced in the late stage of training (Fig. 4), suggesting that the anxiety induced by cocaine can be overcome after several days of cocaine self-administration sessions.

Taken together, results from the present experiment demonstrated that with this self-administration schedule, it took about 7 ~ 8 days of self-administration sessions for animals to exhibit robust lever responses for cocaine and 18 days each of conditioning for animals to show distinctive lever responses for cocaine and saline. Interestingly, their lever pressing pattern closely paralleled their cue reactivity. This is the first study to show the relationship between cue reactivity and subsequent lever pressing for cocaine using laboratory animals. Using these results, an early and late stage of cocaine-associative learning was selected for Experiments 2 & 3 when dopaminergic and behavioral measurements were taking in the presence and absence of cocaine and paired stimuli. Twelve days of conditioning (i.e., 6 days of cocaine and 6 days of saline) was chosen as the early stage of cocaine-associative learning from following reasons: 1) Animals are still acquiring cocaine-taking behavior since they had not yet shown robust lever responses for cocaine, 2) Cocaine-paired stimuli are not yet capable of inducing behavioral changes as assessed by cue reactivity measures, and 3) The number of lever responses for cocaine and saline are comparable with each other. Forty days of a conditioning (i.e., 20 days of cocaine and 20 days of saline) was chosen as the late stage of cocaine-associative learning since 1) Animals were maintaining a high level of cocaine-taking behavior, 2) Cocaine-paired stimuli became capable of eliciting behavioral changes, as assessed by cue reactivity measures, and 3) The number of lever responses for cocaine are significantly more than saline-reinforced responses.

CHAPTER 3. EXPERIMENT 2: EFFECTS OF COCAINE AND SALINE-PAIRED STIMULI ON NACC DA ACROSS DIFFERENT STAGES OF COCAINE-ASSOCIATIVE LEARNING

The purpose of Experiment 2 was to determine whether the influence of cocaine or saline-paired stimuli on NAcc DA levels differs during early or late stages of cocaine-associative learning. Great detail about the neural architecture and a functional role of the NAcc was covered in Chapter 1. Therefore, the following Introduction briefly summarizes why NAcc DA is believed to be an essential component for cocaine addiction and the role of NAcc DA in distinctive stages of cocaine-associative learning.

3-1. INTRODUCTION

3-1-1. Nucleus accumbens medium spiny neurons

NAcc receives major dopaminergic projections from the VTA [54,249]. Major target neurons of the VTA projection are known to be GABAergic medium spiny neurons [33]. The striatal medium spiny neurons also receive excitatory (glutamatergic) inputs from the PFC, thalamus and limbic structures such as hippocampus and amygdala [118]. The axonal varicosities of dopaminergic projections are known to make synaptic contact with dendritic spines of the medium spiny neurons, and are in close proximity to glutamatergic synaptic inputs where synaptic contacts are also formed with medium spiny neuronal dendritic spines (reviewed by [118,273]).

In accordance with the striatal neural architecture, excitatory inputs to the striatum are known to influence “tonic” release of DA in the striatum [45,125,170,185] via action of

glutamate on presynaptic DA varicosities (see review [273]). The excitatory inputs also influence spontaneous activity of the medium spiny neurons to the depolarized "up state" from the hyperpolarized "down state" [33,338]. DA, mainly through stimulation of D1-like receptors, is reported to have inhibitory effects when in the hyperpolarized "down state," while exerting an excitatory effect when the neurons are in the depolarized "up state" [140]. Thus, it was suggested that released DA in the striatum enhances the response to a strong excitatory input while it reduces weak transient input, and thereby increases signal to noise ratio of information reaching to the brain region [140].

Glutamatergic and dopaminergic projections are the major regulator for activity of the medium spiny neurons. It has been suggested that cholinergic interneurons in the striatum innervate medium spiny neurons and regulate action of DA on activity and intracellular signaling cascade of the medium spiny neurons [196]. For example, it has been shown that expression of immediate early gene, *c-fos*, messenger RNAs in the striatal medium spiny neurons induced by the D1 receptor agonist, SKF-82958. *c-fos* expression was augmented by the muscarinic cholinergic receptor antagonist, scopolamine, which by itself did not alter striatal gene expression [325]. Thus, it was suggested that muscarinic receptors in the striatum are involved in the control of striatal D1 receptor stimulation. On the other hand, cocaine was reported to induce increase in *c-fos* expression in the striatal cholinergic interneurons in dose dependent manner [26]. In accordance with the synergistic relationship of dopaminergic and cholinergic inputs, muscarinic (M1 and M4) receptors are reported to be expressed in the medium spiny neurons [6,328] while D1-like and D2-like receptors are expressed in the cholinergic

interneurons [5,196]. These observations suggest that the striatal cholinergic interneurons influence dopaminergic tone and striatal medium spiny neuronal activity, and are involved in exerting cocaine effects.

The GABAergic medium spiny neurons in the striatum are known to send output directly to the substantia nigra pars reticulata and entopeduncular nucleus (internal part of globus pallidus in primates), or indirectly through the subthalamic nucleus and external part of the globus pallidus. Projection neurons in the substantia nigra pars reticulata and entopeduncular nucleus, then send outputs to the brainstem and spinal cord as well as to cortex via mediodorsal thalamus to form a feed back loop to the striatum (see review [33]). Together with the dorsal part of the striatum and globus pallidus, the ventral striatum is known as a major component of basal ganglia. The neural circuit of the basal ganglia is known to involve in response selection, procedural learning and control of behavioral (see review [25]).

Taken together, many excitatory afferents converge in the NAcc. The NAcc together with the dorsal striatum send basal ganglia outputs for executing behavior. Thus, it is thought that the NAcc has an important role in gating or "processing" information reaching the brain region before it sends the "processed" information that prepares the organism to execute the appropriate behavioral output [25]. In fact, the NAcc has been proposed to be a neural interface between limbic and motor system [210]. Because DA in the striatum is reported to enhance signal to noise ratio of excitatory information reaching the striatal medium spiny neurons, a modulatory, rather than a direct role of DA

on behavior is speculated.

3-1-2. Nucleus accumbens, dopamine and cocaine

Cocaine is known to elicit its reinforcing properties by increasing extracellular DA concentration in the NAcc via blockade of DAT [135,183]. The involvement of the mesolimbic dopaminergic system in reinforcing properties of cocaine has been implicated by numerous experiments [117,144,201,236,332]. For example, functional Magnetic Resonance Imaging (fMRI) studies in human cocaine abusers showed that cocaine caused regional signal increases in the NAcc [40], along with cocaine-induced euphoria and craving [40,295]. In experimental animals, pre-treatment with DA antagonists, such as SCH 23390, spiperone [148], and perphenazine [164] are reported to block the reinforcing efficacy of cocaine, as assessed by a dose-dependent alteration in cocaine self-administration response rates [352]. A similar effect was also seen by destruction of NAcc DA-containing neurons with 6-hydroxydopamine (6-OHDA) [259]. These observations support the notion that the mesolimbic dopaminergic system is crucial for the reinforcing effects of cocaine.

3-1-3. Nucleus accumbens, dopamine and cocaine-associated stimuli

Cocaine-paired stimuli are known to trigger craving [88,93,174,213,224], and thereby relapse to cocaine use [324] in cocaine-experienced humans. In experimental animals, cocaine-paired stimuli have also been reported to induce increased locomotor activity [43,106] and reinstatement of cocaine-seeking behavior [277]. Thus it has been

suggested that neutral stimuli present during cocaine taking acquire reinforcing and incentive-motivational property through associative learning [88].

It was postulated that the incentive-motivational property of cocaine-paired stimuli is mediated by the mesolimbic dopaminergic system [106]. For example, it was shown that cocaine-paired stimuli increased plasma concentrations of homovanillic acid (HVA), a major DA metabolite, along with increased subjective feelings of craving in drug-experienced individuals [180]. The increased craving was sufficiently blocked by treatment with D2-like receptor antagonist haloperidol [22]. In experimental animals, however, cocaine-seeking behavior induced by cocaine-paired stimuli was suppressed by DA receptor antagonists [7,65,70], and by DA agonists [7,71], thus obscuring the role of DA in the incentive motivational aspect of cocaine-paired stimuli. Moreover, observations from *in vivo* microdialysis measurement of DA levels in the NAcc suggested that increased DA levels in the NAcc are not always necessary for behavioral expression to cocaine-paired stimuli [42,215]. These observations suggest the necessity of reconsiderating the role of NAcc DA in incentive motivational aspects of cocaine-paired stimuli.

3-1-4. Midbrain dopamine neurons and associative leaning

Because DA transmission is known to initiate intracellular signaling cascades [301] that are largely implicated in learning and memory [1,36,51,73,110,130,132,141,151,195,217,288,350], there has been considerable interest in the involvement of DA in learning processes. Using natural rewards such as food and

drink, electrophysiological studies in monkeys illustrated the role of midbrain DA neurons in associative learning [145,188,273,274]. During initial stages of learning, the midbrain DA neurons show activation [295], an effect that becomes less prominent as animals learn to predict which response would trigger a reward [188]. After sufficient training, the presentation of a predicted reward no longer elicits responses in the DA neurons [145]. Similar observations were seen using *in vivo* microdialysis and voltammetric measurement of DA turnover in the NAcc [12,82,253].

Conditioned stimuli are reported to be less effective in eliciting neural activation than primary rewards [273], though response patterns of DA neurons to conditioned and primary rewards are similar [188,207,273,275]. The activity initially elicited by a primary reward is reported to gradually transfer to a conditioned stimulus that predicts availability of the primary reward [188,251,273,275]. An *in vivo* voltammetry study in rats lever-pressing for foods also showed similar DA signal transduction in response to food reward and to stimulus associated with food reward [253]. However, extensive over-training attenuates the responsiveness of DA neurons to conditioned stimuli [188,251].

DA neurons are reported to respond to appetitive stimuli, and also to novel [188] and salient stimuli [147]. *In vivo* microdialysis studies showed that aversive stimuli also increases NAcc DA levels [2,270,355]. Thus, it is plausible that the mesolimbic DA serves as a neural modulator for intensifying stimuli saliency to help form proper responses to environmental stimuli. In this way, DA may be involved in guiding

associative learning. This view is consistent with striatal neural architecture, in which the NAcc is an interface between limbic and motor system [210], and consistent with a modulatory role of DA that enhances signal to noise ratio of excitatory inputs to the striatal medium spiny neurons. Midbrain DA is involved in early stages of learning but not in late stages. Thus, once performance is automatized via repeated learning trials, the basal ganglia circuit may able to prepare organisms for proper stimulus-response without dopaminergic signals from the midbrain.

3-1-5. Nucleus accumbens dopamine in drug addiction and abnormal learning process

The midbrain DA neuronal activity appears to be restricted to an early stage of appetitive-associative learning. On the other hand, the mesolimbic dopaminergic system is known to be persistently activated by drugs of abuse [235,236,295], possibly leading to strengthened drug-stimulus associations [295]. Little research, however, has investigated the role of the mesolimbic dopaminergic system in distinctive stages of cocaine-associative learning.

Does the mesolimbic dopaminergic system respond to cocaine-paired stimuli during early stages of learning and become less responsive after extensive conditioning? Or does the mesolimbic dopaminergic system show a persistent response even after extensive conditioning because of the persistent stimulation of the dopaminergic system by cocaine? In order to determine such dynamics of the NAcc DA transmission during

drug-associative learning processes, *in vivo* microdialysis was performed on animals that underwent either a limited or protracted self-administration conditioning sessions.

3-2. MATERIALS AND METHODS

Subjects: Male albino Sprague-Dawley rats (Animal Resource Center, Austin, TX) weighing approximately 250 – 300 g at the beginning of the experiments were used (N = 66). The animals were group-housed and maintained on a 12 hr. reversed light/dark cycle (light on 7:00 p.m. to 7:00 a.m.). They were handled daily at least one week prior to the experiment and continuously handled throughout the experiment. Food and water were available *ad libitum* in the home cage except during food training.

Apparatus: Food training, *in vivo* microdialysis test sessions, and self-administration sessions were conducted in identical one-lever operant chambers constructed of Plexiglas located in sound-attenuating chambers as described in the Experiment 1.

Surgery: Prior to surgery, animals were trained to lever press for food on a Fixed Ratio 1 (FR1) schedule of reinforcement as described in Experiment 1. After the completion of the food training, animals were implanted with a chronic silastic intravenous jugular catheter (see Methods, Experiment 1). Animals were also stereotaxically implanted with an unilateral guide cannula (21 ga) aimed to 6.3 mm above the NAcc (Tooth bar = 5.0 mm above zero, AP: 3.0 mm; ML: ± 1.7 mm; DV: 2.5 mm) according to the atlas of Paxinos and Watson (1997) [232]. Left versus right NAcc placements were counterbalanced across animals. These animals underwent a minimum of one-week

recovery prior to the beginning of the experiment. Catheter patency was maintained by infusing one U/ml streptokinase and 30 U/ml heparin mixed in 0.1 cc of isotonic saline as described in Experiment 1.

Cocaine/Saline Conditioning Self-administration Sessions: Over the course of conditioning sessions, animals had alternating days of cocaine and saline availability during one-hour daily self-administration sessions (e.g., 6 days of cocaine and 6 days of saline sessions = Limited training, or 20 days of cocaine and 20 days of saline sessions = Protracted training). In the first 30 min of these sessions, the chamber was darkened and the lever was retracted for habituation to the chamber as described in Experiment 1. After the habituation period, the house light illuminated, the visual and olfactory environmental cues (either black or white felt "walls" attached to the sides of the clear Plexiglas operant chamber and a cinnamon or rose oil-based scents saturated on a cotton ball and placed under the grid floor of the operant chamber) were introduced into the operant chamber, the lever protruded, and cocaine or saline then became available for 30 min. During these sessions, each lever press resulted in the delivery of 0.5 mg/kg/0.1 cc cocaine hydrochloride mixed in isotonic saline (cocaine days), or saline alone (0.1 cc) (saline days), infused over 6 seconds. After each infusion, there was a 20-sec "time-out" period, during which time the lever was retracted, the stimulus light is off and no infusions could be delivered. "Pseudo-conditioning" and "cocaine-naïve" groups were included to assess the effects of cues without conditioning and cocaine alone, respectively. The "pseudo-conditioning" group underwent the same number of conditioning sessions as the Limited training groups (i.e., a total of 12 days conditioning

sessions) but the cues were presented randomly during the self-administration sessions. In this group, the visual cues (black or white walls) and the olfactory cues (cinnamon or rose scent) were randomly assigned to each day of the 12 daily conditioning sessions. The "cocaine naïve" animals were also placed into the operant chamber for the same number of days as the limited conditioning groups but they were removed from the chamber after the 30-min habituation period and before cocaine or saline became available. Failure to reach a criterion responses (mean responding ≤ 1) for cocaine day sessions resulted in exclusion from the experiment, since animals insensitive to a primary reinforcer could not be expected to develop conditioned reinforcement [75].

In Vitro Recovery Calibration: Prior to probe recovery, all probes were flushed with nanopure water. At the day of probe calibration, 1.0 ml gastight Hamilton 1000 series syringes were filled with freshly prepared filtered Ringer's solution (128.3 mM NaCl, 1.35 mM $CaCl_2$, 2.68 mM KCl, and 2.0 mM $MgCl_2$), and pumped through the probe at 1.63 μl/min, with the probe tips in a beaker containing the Ringer's solution, ascorbate (0.001%), and 20 nM DA, maintained at 37° C. Degradation of DA was prevented by adding perchloric acid solution (1M Na Bisulfite and 0.2 M EDTA in 0.05 N $HClO_4$) into collecting tubes. Ten-min samples from each probe were collected and assayed by high performance liquid chromatography (HPLC) with electrochemical detection (HPLC-EC). Probe recoveries were calculated by comparing the peak heights of samples to those from a standard (5 nM DA). The recovery of probes used in the experiment (expressed as mean ± SEM) was 15.17 ± 0.35 % (N = 66).

Microdialysis probe implantation: After completion of all self-administration sessions, animals were briefly anesthetized with ultrashort-acting barbiturate, methohexital sodium (Brevital®, approximately 6 mg/i.v.), and implanted with a microdialysis probe through the previously implanted guide cannula. Each microdialysis probe was connected to a 1.0 ml gastight Hamilton 1000 series syringe mounted on a syringe pump (Razel®, Model A), and freshly prepared Ringer's solution was pumped through the probe. Animals implanted with the probe remained in a holding chamber overnight with the syringe pump speed set at 0.261 μl/min. Bedding, food, and water were available in the holding chamber. Thirty min prior to the test session, the pump speed was changed to 1.63 μl/min.

Test Day conditions: At least 12 hours after the probe implantation, animals were tested for locomotor and dopaminergic responses before and after a self-administered infusion of either cocaine (3.0 mg/kg) or saline alone (0.1 cc). The intravenous dose of cocaine was chosen because it resulted in an approximately 300 % increase in the NAcc DA levels from basal levels in male Sprague-Dawley rats [150] similar to % increase achieved by intraperitoneal (i.p.) cocaine administration of 10 ~ 15 mg/kg [29,168], an i.p. dose widely used in conditioning studies [43,106,108]. Animals were placed in the darkened operant chamber as during the training sessions, with the lever retracted for the first 30 min. After the 30 min, the house light illuminated, the olfactory and the visual environmental cues were introduced into the operant chamber, and the lever extended into the chamber. For animals tested with cocaine-paired stimuli ("CS" group), the olfactory and visual cues paired with cocaine training days were present. For animals

tested with saline-paired stimuli ("SS" group), testing was conducted with the cues paired with saline-training days. For "pseudo conditioning" group ("RS" group), testing was conducted with one pair of the olfactory and the visual cues randomly presented during the conditioning sessions. After the animal pressed the lever, 0.1 cc of 3.0 mg/kg cocaine or saline was intravenously delivered over 6 seconds. Brain dialysate and locomotor activity data were collected in 10-min sample bins over the course of the one-hour session; 3 baselines during the 30 min habituation periods and 3 post self-infusion samples. The average of baseline DA levels was defined as 100 % and individual samples were represented relative to 100 %. Locomotor activity was recorded via computer assessment of photobeam interruptions inside the operant chamber.

Assay of dialysate: The dialysates were analyzed for DA concentrations using HPLC equipped either with **a)** ESA Catecholomine HR80 reverse-phase column or Rainin microsorb-MV C-18 RP column, ESA Model 5200A Coulochem II Detector, a Model 5020 Guard Cell and a Model 5014B Choulmetric Analytical Cell or **b)** Shizeido capcell C-18 narrow bore column, ESA Model 5200 A Coulochem II Detector, a Model 5020 Guard Cell and a Model 5041 Amperometric Analytical Cell. For the HPLC set up **a)**, the mobile phase contained 82.4 mM $Na_2H_2PO_4$, 2.23 mM EDTA, 7.5 ~ 8.0 % (v/v) acetonitrile, and 0.46 ~ 2.08 mM 1-octanesulfonic acid, pH 5.5, analytical cell potential was set at + 20 ~ + 220 mV (oxidation) and at – 75 ~ –175 mV (reduction), and pump speed at 0.5 ml/min. The detection limit for DA was approximately 0.3 pg/sample. For the HPLC set up **b)**, the mobile phase contains 150 mM $Na_2H_2PO_4$, 50 µM EDTA, 4.5 mM ~ 6.0 mM Sodium Dodecyl Sulfate, 4.76 mM Citric Acid, 10 ~ 15 % (v/v)

Acetonitrile, 10 ~ 15 % methanol, pH 5.6, an analytical cell potential was set at + 200 mV (oxidation), and pump speed at 0.2 ml/min. The detection limit for DA was approximately 0.1 pg/sample. Data are collected and analyzed using an ESA Model 500 Data station.

Histological analysis: After the experiment, animals were euthanized by administering an overdose of sodium pentobarbital (Nembutal) and brains were removed and stored in 10 % formaldehyde solution with 30 % sucrose. The probe placements of each animal were verified from coronal sections (48 µm) stained with cresyl violet using the atlas of Paxinos and Watson (1997) [232]. Animals in which active membrane region outside of NAcc were excluded from further analysis. Fig. 5-A & 5-B shows the probe traces in the coronal sections. Fig. 6 shows a dialysis probe trace from a representative animal. This study was not intended to differentiate the "core" and "shell" subterritories of the NAcc. Because of the increasing interest in the subterritories however, the best estimate was made in which the active membrane region inside of each sub territory (Table 1). One animal died unexpectedly after the experiment, so the histological analyses were not performed on the animal.

Data analysis: Dialysate DA in the Limited and Protracted training groups were analyzed separately by a two-way ANOVA (Condition x Time) with repeated measures on Time. When there were main effects or interactions in the overall analysis, *Post hoc* analyses (Fishers LSD) were used to detect significant differences at specific time points.

3-3. RESULTS

Lever responses during the cocaine self-administration sessions: The mean number (mean ± SEM) of lever responses for cocaine from animals that underwent the limited training (N = 36) were 5.23 ± 1.60, 6.32 ± 1.47, and 5.77 ± 1.15 for "Pseudo-conditioning (RS)," animals tested with cocaine-stimuli ("CS"), and animals tested with saline-stimuli ("SS"), respectively. The corresponding cocaine amounts for the mean lever responses were 2.62 ± 0.80 mg/kg, 3.16 ± 0.74 mg/kg, and 2.89 ± 0.58 mg/kg for "RS", "CS", and "SS" group, respectively. A one-way ANOVA showed that the amount of cocaine self-administered during the training sessions were comparable between groups [F (2,33) = 0.1021; NS].

The mean number (mean ± SEM) of lever responses for cocaine from animals that underwent the Protracted training (N = 33) were 6.94 ± 0.78 for "CS" and 6.66 ± 0.79 for "SS." The corresponding cocaine amounts for the mean lever responses were 3.47 ± 0.39 mg/kg for "CS" group and 3.33 ± 0.40 mg/kg for "SS" group. A t-test showed no significance difference in amount self-administered prior to the test session between groups [t (21) = 0.2492; NS].

Effect of paired cues on NAcc DA levels from Limited training animals: Fig. 6-A shows the DA response at the test session from following groups that underwent Limited training. **1)** Animals a received self-injection of cocaine (3.0 mg/kg) in the presence of cocaine-paired stimuli ("Cocaine + CS", n = 10). **2)** Animals received a self-injection of cocaine in the presence of saline-paired stimuli ("Cocaine + SS", n = 9). **3)** A "Pseudo-

conditioning" group that received a self-injection of cocaine in the presence of randomly conditioned stimuli ("Cocaine + RS", n = 5). **4)** "Cocaine naïve " group that received a self-injection of cocaine first time at the test session ("Acute", n = 7). The mean (mean ± SEM) baseline DA concentration was 1.15 ± 0.16 nM, 0.52 ± 0.12 nM, 0.62 ± 0.18 nM, and 0.65 ± 0.22 nM for "Cocaine + RS", "Cocaine + CS", "Cocaine + SS", and "Acute," respectively. Although the baseline DA concentration of "Cocaine + RS" was higher than other groups, a one-way ANOVA revealed that baseline DA levels of these groups were not significantly different each other [$F(3,27) = 2.0494$; N.S.].

A significant portion of the probe was immersed into the "core" than into the "shell" ($p < 0.01$, Table 1). There were no significant differences in the amount of the active probe membrane region immersed in the subterritories between groups. " A two-way ANOVA revealed no effect of Group [$F(3,26) = 0.9961$; N.S.], a significant effect of Subterritory [$F(1,26) = 54.1371$; $p < 0.01$] and no Group X Subterritory interaction [$F(3,26) = 0.7008$; N.S.].

DA levels were significantly increased after the cocaine infusion in all groups ($p < 0.05$ for "Cocaine + CS" and "Cocaine + SS", $p < 0.01$ for "Cocaine + RS" and "Acute"). A two-way ANOVA (Group X Time) with repeated measures on the Time factor revealed no effect of Group [$F(3,27) = 2.0239$; N.S.], but a significant effect of Time [$F(5,135) = 36.2454$; $p < 0.01$] and a significant effect of Group by Time interaction [$F(15,135) = 1.7736$; $p < 0.05$]. *Post hoc* tests revealed that the cocaine-induced increase in DA levels of "Cocaine + RS" and "Acute" were significantly higher than those observed in

"Cocaine + CS" and "Cocaine + SS" at the first 10 min interval after the cocaine infusion ($p < 0.01$). The cocaine-induced increase in DA level of "Cocaine + CS" was also significantly higher than that of "Cocaine + SS" ($p < 0.05$).

Fig. 7-A shows the DA response at the test session from animals that underwent the Limited training and tested with **1)** a self-injection of saline in the presence of cocaine-paired stimuli ("Saline + CS", n = 6) and **2)** a self-injection of saline in the presence of saline-paired stimuli ("Saline + SS", n = 6). The mean (mean± SEM) baseline DA concentration was 1.35 ± 0.24 nM for "Saline + CS" and 0.78 ± 0.17 nM for "Saline + SS." Although the baseline concentration of "Saline + CS" was higher than that of "Saline + SS, " a t-test showed that the difference was not significant [t (10) = 1.958; NS].

A significant portion of the probe was immersed into the "core" than into the "shell" ($p < 0.01$, Table 1). There were no significant differences in the amount of the active probe membrane region immersed into the subterritories between groups." A two-way ANOVA revealed no effect of Group [F (1,10) = 0.2135; N.S.], a significant effect of Subterritory [F (1,10) = 82.649; $p < 0.01$] and no Group X Subterritory interaction [F (1,10) = 2.9297; N.S.].

When animals received a self-infusion of saline, DA levels from animals tested with cocaine-paired stimuli significantly depressed from their basal levels ($p < 0.01$) while DA levels of animals tested with saline-paired stimuli did not change. The post-infusion DA

levels of the animals tested with cocaine-paired stimuli were also significantly lower than those observed in the animals tested with saline-paired stimuli. A two-way ANOVA (Group X Time) with repeated measures on the Time factor showed significant effects of Group [$F(1,10) = 17.6327$; $p < 0.01$], Time [$F(5,50) = 5.7738$; $p < 0.01$] and a Group by Time interaction effect [$F(5,50) = 6.123$; $p < 0.01$]. *Post hoc* tests revealed that the DA levels of post-saline infusion from "Saline + CS" were significantly lower than those observed in "Saline + SS" in all time points ($p < 0.01$ and $p < 0.05$).

Effect of paired cues on locomotor activity from Limited training animals: Fig. 6-B shows locomotor activity from animals that self-administered cocaine on the test day after Limited training. Following the self-injection of cocaine, locomotor activity increased significantly in the "Cocaine + CS" ($p < 0.01$),"Cocaine + SS" ($p < 0.01$) and "Cocaine + RS" ($p < 0.05$), but not in the "Acute" group. A two-way ANOVA (Group X Time) with repeated measures on the Time factor revealed no effect of Group [$F(3,27) = 0.1957$; NS], a significant effect of Time [$F(5,135) = 14.7587$; $p < 0.01$] and no Group by Time interaction [$F(15,135) = 0.8707$; NS]. *Post hoc* test revealed that the cocaine-induced increase in locomotor activity of "Cocaine + CS" and "Cocaine + SS" was significantly higher than those observed in "Acute" animals at first post-infusion 10 min interval ($p < 0.05$).

When animals received a self-infusion of saline, their locomotor activities were compatible between groups ("Saline + CS" and "Saline + SS," Fig. 7-B). A two-way ANOVA (Group X Time) with repeated measures on the Time factor showed no

significant differences in Group [F (1,10) = 0.1491; NS], Time [F (5,50) = 0.7968; NS] and no significant Group by Time interaction effects [F (5,50) = 0.1536; NS].

Effect of paired cues on NAcc DA levels after Protracted training: Fig. 8-A shows the % increase of DA levels at the test session from the following groups that underwent the Protracted training: **1)** Animals that received a self-injection of cocaine (3.0 mg/kg) in the presence of cocaine-paired stimuli ("Cocaine + CS," n = 6) and **2)** animals that received a self-injection of cocaine in the presence of saline-paired stimuli ("Cocaine + SS," n = 6). The mean (mean ± SEM) baseline concentration of their DA levels were 0.58 ± 0.18 nM and 0.63 ± 0.17 nM in "CS" and "SS," respectively. A t-test revealed that there was no significant difference in their baseline DA levels between groups [t (10) = -0.2351; NS].

A significant portion of the probe was immersed into the "core" than into the "shell" ($p < 0.01$, Table 1). There were no significant differences in the amount of the active probe membrane region immersed into the subterritories between groups." A two-way ANOVA revealed no effect of Group [F (1,10) = 0.3553; N.S.], a significant effect of Subterritory [F (1,10) = 49.8889; $p < 0.01$] and no Group X Subterritory interaction [F (1,10) = 1.3203; N.S.].

The cocaine infusion induced a significant DA increase in both "Cocaine + CS" and "Cocaine + SS" ($p < 0.01$ and $p < 0.05$). A two-way ANOVA (Group X Time) with repeated measures on the Time factor revealed a no significant Group effects [F (1,10) =

0.2787; NS], a significant effect of Time [F (5,50) = 7.1174; $p < 0.01$], and no significant Group by Time interaction effects [F (5,50) = 0.2872; NS]. *Post hoc* tests revealed that the cocaine-induced increase in DA levels of animals tested with cocaine-paired stimuli were comparable to those tested with saline-paired stimuli.

Fig. 9-A shows the DA response at the test session from animals that received a self-injection of saline in presence of either **1)** cocaine-paired stimuli ("Saline + CS," n = 5) or **2)** saline-paired stimuli ("Saline + SS," n = 6). The mean (mean ± SEM) baseline concentration of their DA levels were 1.29 ± 0.41 nM and 1.24 ± 0.36 nM in "Saline + CS" and "Saline + SS," respectively. A t-test showed no significant differences in baseline DA levels between groups [t (9) = 0.0923; NS].

There were no significant differences in portion of the probe immersed into the "core" and the "shell" (Table 1). A two-way ANOVA revealed no effect of Group [F (1,9) = 0.9898; N.S.], or Subterritory [F (1,9) = 2.3536; N.S.] and no Group X Subterritory interaction [F (1,9) = 0; N.S.].

A two-way ANOVA (Group X Time) with repeated measures on the Time factor revealed no significant Group effects [F (1,9) = 0.003; NS], but a significance effect of Time [F (5,45) = 8.8646; $p < 0.01$] and no significant Group by Time interaction effects [F (5,45) = 0.8281; NS]. *Post hoc* tests revealed that the saline infusion resulted in significant depressions of DA levels from their basal levels in both groups at 20-min and 30-min

post-infusion sampling time points ($p < 0.01$ and $p < 0.05$). However, the post-infusion DA levels between groups were comparable.

Effect of paired cues on locomotor activity from Protracted training animals: Fig. 8-B shows locomotor activity on the test day from animals that underwent Protracted training. Following the self-injection of cocaine, their locomotor activity increased significantly in both "Cocaine + CS" and "Cocaine + SS." A two-way ANOVA (Group X Time) with repeated measures on the Time factor revealed no effect of Group [$F(1,10) = 1.3939$; NS], but significant effects of Time [$F(5,50) = 14.187$; $p < 0.01$] and no Group X Time interaction [$F(5,50) = 0.8227$; NS]. *Post hoc* tests revealed that the cocaine-induced locomotor hyperactivity of "Cocaine + CS" was significantly higher than that observed in "Cocaine + SS" during the first10-min post-cocaine interval ($p < 0.05$).

Locomotor activity of animals receiving a self-infusion of saline at the test session ("Saline + CS" and "Saline + SS") was comparable (Fig. 9-B). A two-way ANOVA (Group X Time) with repeated measures on the Time factor showed no significant Group [$F(1,9) = 1.4427$; NS], Time [$F(5,45) = 0.9377$; NS] or Group X Time interaction effects [$F(5,45) = 0.2977$; NS].

Limited conditioning						
Testing Drug	Cocaine			Saline		
Testing Environment	"CS"	'SS"	"RS"	"Acute"	'CS"	"SS"
Core Shell	1.03 ± 0.12 ** 0.19 ± 0.10	1.29 ± 0.11** 0.50 ± 0.19	1.18 ± 0.27** 0.20 ± 0.20	1.06 ± 0.16** 0.52 ± 0.19	1.00 ± 0.18** 0.12 ± 0.08	0.96 ± 0.19** 0.36 ± 0.17

Protracted conditioning				
Testing Drug	Cocaine		Saline	
Testing Environment	"CS"	"SS"	"CS"	"SS"
Core Shell	1.08 ± 0.11** 0.53 ± 0.18	1.30 ± 0.08** 0.53 ± 0.17	1.10 ± 0.14 0.92 ± 0.13	0.89 ± 0.17 0.72 ± 0.19

Table 1. Estimated (mean ± SEM, mm) active membrane region of the probe inside of the NAcc "core" and "shell" subterritories. In all testing conditions except animals that underwent protracted conditioning and tested with a self-infusion of saline, the amount of active membrane region of the probe was significantly more in the "core" than in the "shell." ** represents $p < 0.01$. There is no significant difference in the amount between groups in all testing conditions.

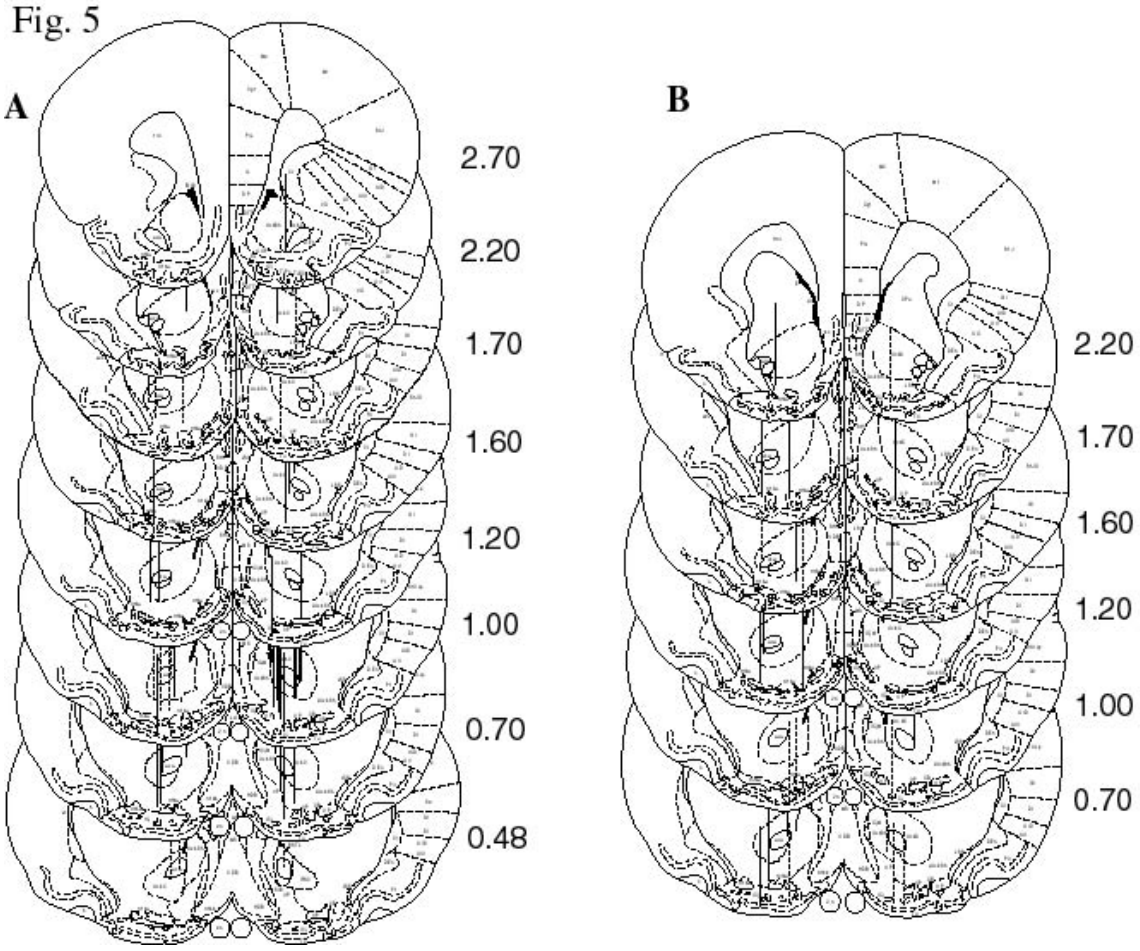

Fig. 5. Schematic representation of an active dialysis probe membrane region in the NAcc of animals that completed the dialysis experiment with **(A)** the limited conditioning (N = 43) and **(B)** the protracted conditioning (N = 23). The numbers depicted next to each brain slice indicate the mm anterior to bregma. The diagram was drawn with the assistance of the atlas of Paxinos and Watson (1997) [232]. Solid lines indicate animals that were tested with a self-infusion of cocaine. Dotted lines indicate animals that were tested with a self-infusion of saline.

Fig. 6.

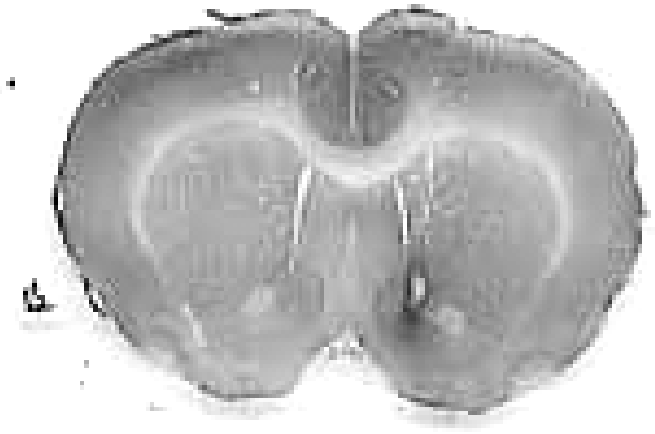

Fig. 6. A dialysis probe trace in the NAcc from a coronal section (48 µm) of a representative animal. Width is 681 % and Height is 667 % of actual size. Note that the trace in the section is shorter than the actual length of the active probe membrane region since the coordinate for the probe implantation was angled (Tooth bar = 5.0 mm above zero) in order to increase likelihood to hit the NAcc.

Fig. 7

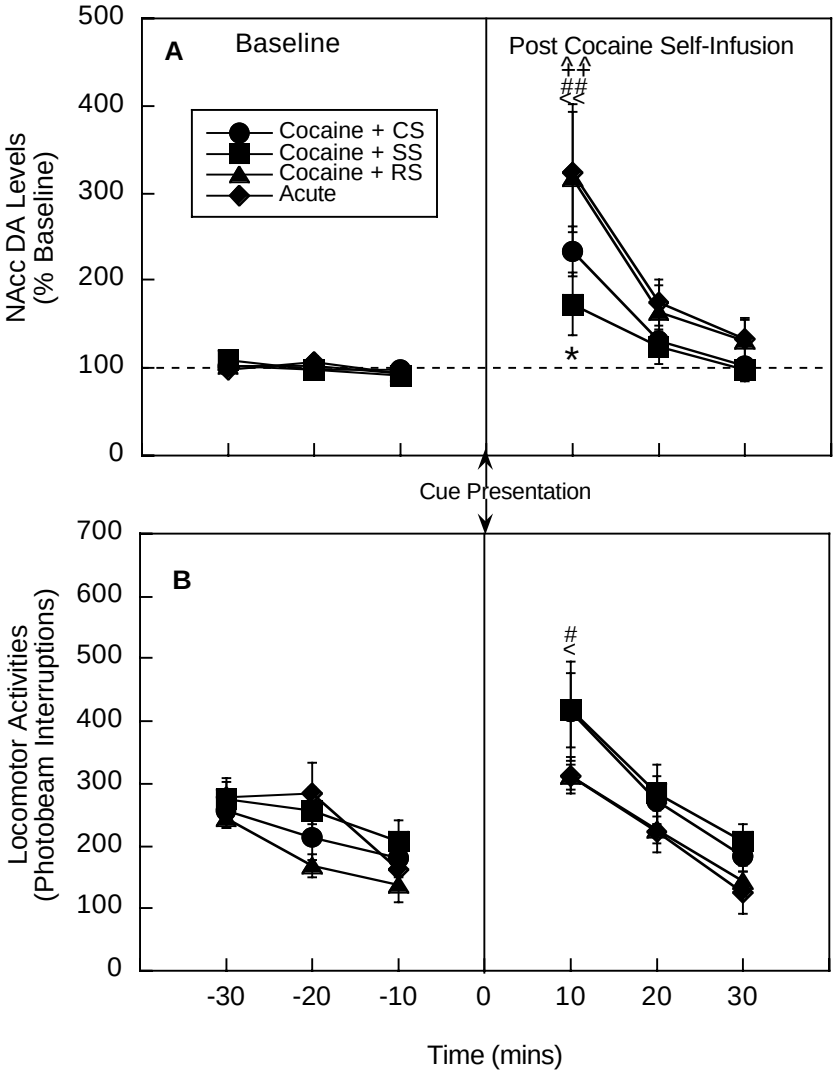

Fig. 7. Effect of paired cues and cocaine self-infusion on **(A)** NAcc DA levels and **(B)** locomotion from the Limited training animals (● = Cocaine + CS, ■ = Cocaine + SS, ▲ = Cocaine + RS, and ◆ = Acute). **(A)** Cocaine + RS and Acute had a greater increase in DA levels than Cocaine + CS and Cocaine + SS (++, ^^ = $p < 0.01$ in Cocaine + CS and Cocaine + SS versus Cocaine + RS, respectively; ##, << = $p < 0.01$ in Cocaine + CS and Cocaine + SS versus Acute, respectively). The increase in DA levels was greater in Cocaine + CS than Cocaine + SS, * = $p < 0.05$). **(B)** Locomotor activity increased significantly in Cocaine + CS ($p < 0.01$). Cocaine + SS ($p < 0.01$) and Cocaine + RS ($p < 0.05$). The increase was significantly lower in Acute compared to Cocaine + CS and Cocaine + SS (#, < = $p < 0.05$ in Cocaine + CS and Cocaine + SS versus Acute, respectively).

Fig. 8

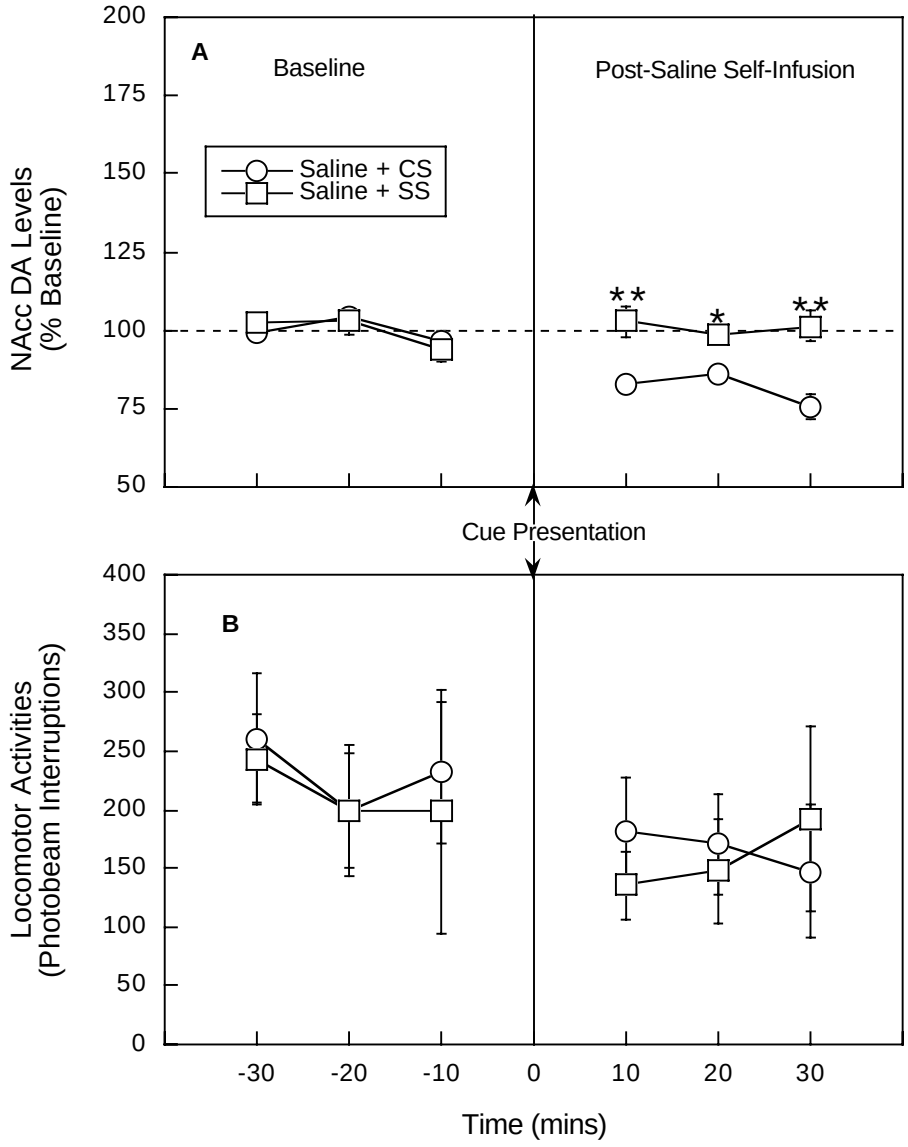

Fig. 8. Effect of paired cues with a self-infusion of saline on **(A)** NAcc DA levels and **(B)** behavioral responses from the limited training animals (○ = "Saline + CS," □ = "Saline + SS"). **(A)** A self-infusion of saline significantly depressed DA levels in "Saline + CS" ($p < 0.01$) whereas DA levels of "Saline + SS" did not change overtime. The post-infusion DA levels of "Saline + CS" were also significantly lower than "Saline + SS" (**. * represent $p < 0.01$ and $p < 0.05$). **(B)** There was no overall significant difference in their locomotor activity.

Fig. 9

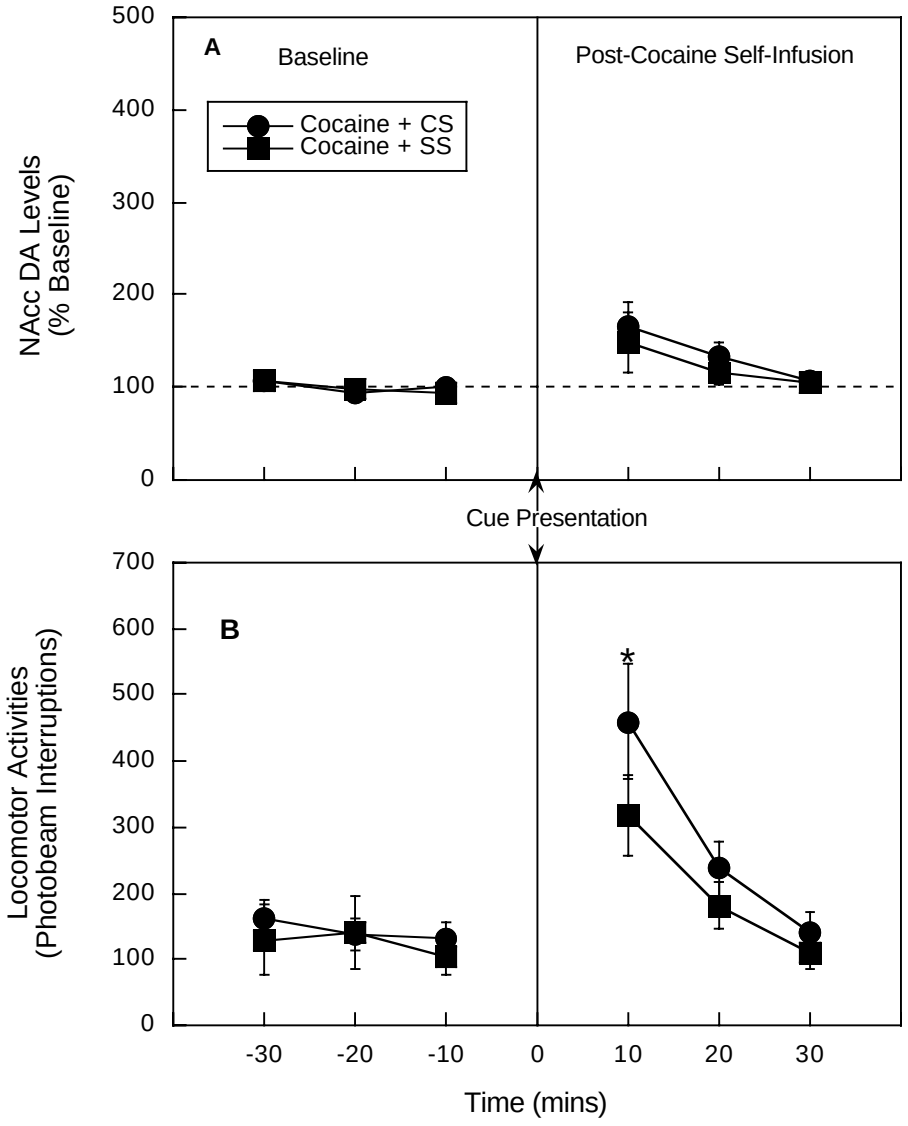

Fig. 9. Effect of paired cues with a self-infused cocaine on **(A)** NAcc DA levels and **(B)** behavioral responses from the protracted training animals (● = "Cocaine + CS," ■ = "Cocaine + SS"). **(A)** DA levels increased significantly during the first10 min post-cocaine infusion in both groups ($p < 0.01$). However, there was no overall significant difference between groups. **(B)** Cocaine induced a significant increase in locomotor activity in both groups. The cocaine-induced locomotor hyper activity in "Cocaine + CS" during the first10-min post-cocaine interval was significantly higher than "Cocaine + SS" (*, $p < 0.05$).

Fig. 10

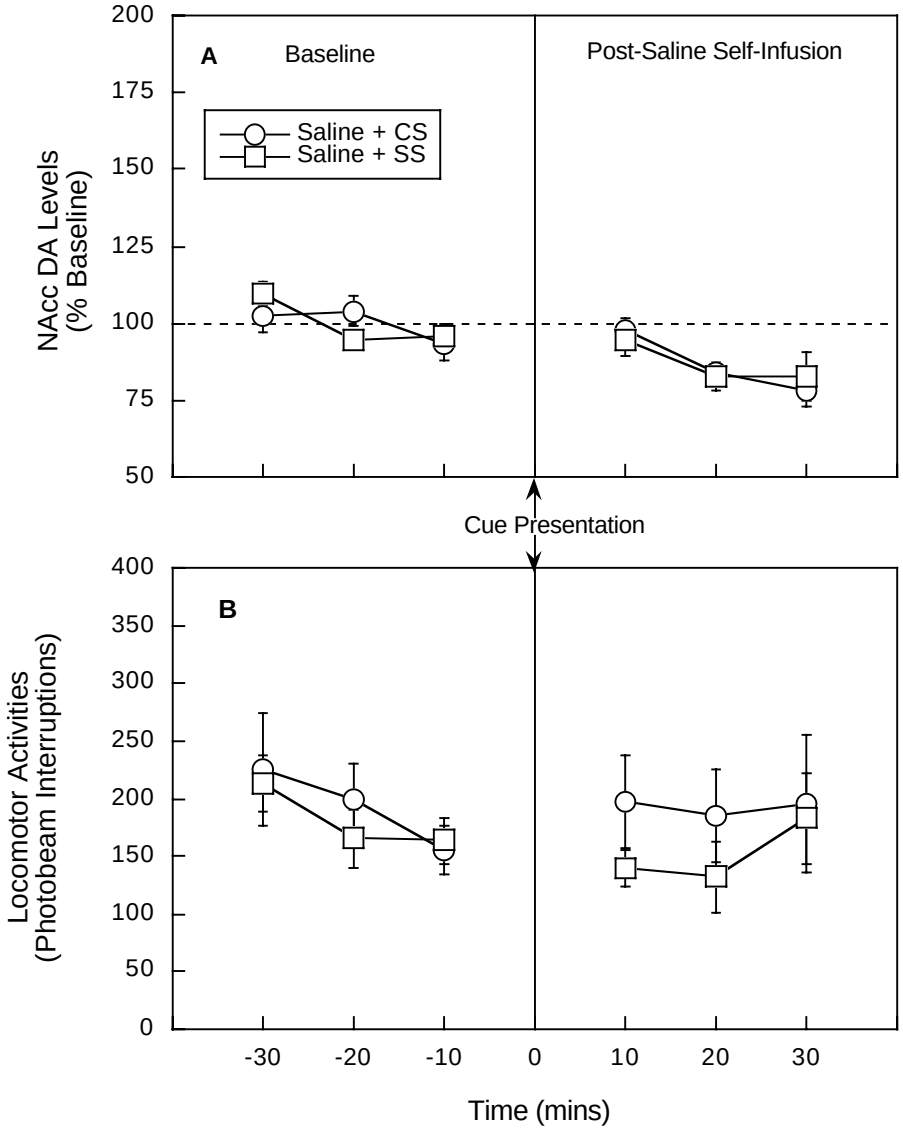

Fig. 10. Effect of paired cues with a self-infused saline on **(A)** NAcc DA levels and **(B)** behavioral responses from the protracted training animals (○ = "Saline + CS," □ = "Saline + SS"). **(A)** A saline infusion significantly decreased DA levels in both groups at 20- and 30-min post-infusion sampling bins ($p < 0.01$). There was no overall significant difference between groups across all time points. **(B)** There was no overall difference in their locomotor activity between groups.

Fig. 11

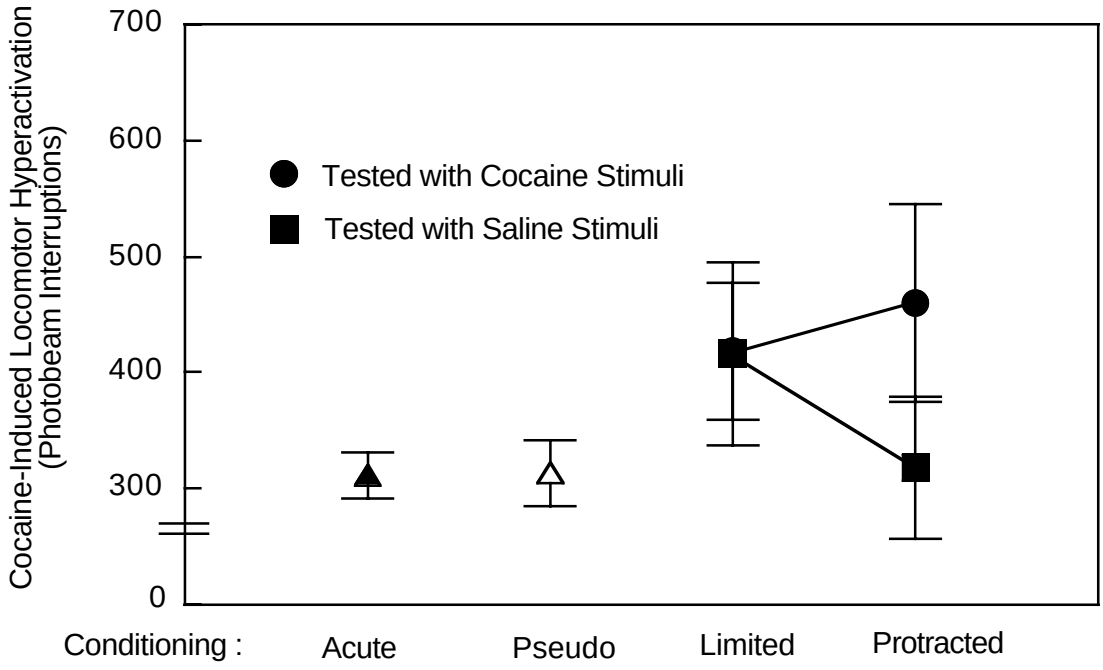

Fig. 11. The first 10-min sampling of cocaine-induced locomotor hyperactivation from limited and prolonged conditioned groups (▲ = "Acute," △ = "Cocaine + RS," ● = " Cocaine + CS," ■ = "Cocaine + SS"). The protracted conditioning resulted in augmentation of the cocaine-induced hyperactivity with cocaine-paired stimuli and suppression of the cocaine-induced hyperactivity with saline-paired stimuli.

3-4. DISCUSSION

The major finding of the present study is that the influence of cocaine and cocaine-conditioned stimuli on the NAcc DA levels decayed with protracted conditioning. During early stages of training, cocaine-induced DA increase was significantly different between animals tested with cocaine-paired stimuli and those tested with saline-paired stimuli (Fig. 7-A). Since the amount of cocaine self-administered by these animals prior to the test session is comparable between groups, the difference between groups was likely induced by the presence of the conditioned stimuli. Differences in NAcc DA turnover in response to saline and cocaine-conditioned stimuli disappeared with Protracted conditioning (Fig. 9-A). Results from Experiment 1 showed that during the early stage of training, when animals were still acquiring cocaine-taking behavior, cocaine-paired stimuli were not yet capable of inducing behavioral hyperactivity. In the late stage of training, when animals were in a maintenance phase of cocaine-taking, cocaine-paired stimuli became capable of eliciting behavioral hyperactivity. The obvious differences seen in this microdialysis study were due to the amount of training and thus the distinctive stage of cocaine-associative learning. The results are in accordance with findings of others that utilized conventional rewards [145,188,273,274], and indicate that changes in NAcc DA levels induced by cocaine and conditioning is also dependent on particular stages of learning.

Animals that underwent protracted conditioning showed that conditioned stimuli-induced behavioral sensitization can occur in the absence of differences in NAcc DA levels (Fig

9-A & 9-B). These results are consistent with reports that the acquisition process of stimulant-induced context-dependent behavioral sensitization is more vulnerable to DA receptor blockade than is the expression of already established sensitization behavior [15,16]. Taken together, these results further suggest that the involvement of DA in distinctive stages of cocaine-associative learning can be dissociated from behavior. Such learning state-dependent influences of conditioned stimuli on NAcc DA levels may be one reason why some studies found NAcc DA increases in the presence of cocaine-paired stimuli [90,91,106,108,117,179,214] while others did not [42,43,65,215].

The present study showed that cocaine-paired stimuli augmented the cocaine-induced locomotor hyperactivation in animals that underwent the protracted conditioning (Fig. 9-B). It has been suggested that conditioning is one of factors contributing development of "behavioral sensitization" to psychomotor stimulant [234], thereby known as context-dependent behavioral sensitization. For example, cocaine-induced locomotor hyperactivation has been found to be more prominent when animals were tested in the environment associated with previous cocaine exposure [245] or when stimuli previously paired with cocaine is present [143]. However, comparison of cocaine-induced locomotor hyperactivation from our limited and protracted conditioning groups (Fig. 11) revealed that the quantitative difference in locomotor hyperactivation induced by cocaine was not solely due to the context-dependent behavioral sensitization. Cocaine-induced locomotor hyperactivation was suppressed with the presence of saline-paired stimuli, which attributed to the quantitative difference. Thus, expectation elicited by the

conditioned stimuli greatly influences the expression of cocaine-induced behavioral activity.

The “pseudo-conditioning” group, in which stimuli had been randomly presented during conditioning sessions, exhibited comparatively low activity levels after cocaine infusion (Fig. 7-B). The behavioral response of the “pseudo-conditioning” animals to cocaine was almost identical to cocaine-naïve animals that had no conditioning experience. This finding could be due to anxiogenic properties of cocaine. For instance, stressful events have been reported to suppress locomotor activity in rodents [81,348,353]. Cocaine is known to have “conflicting” effects, both anxiogenic and reinforcing in nature. The anxiogenic properties of cocaine have also been reported in humans [99]. Our results from the Experiment 1 suggest that it takes several days of self-administration sessions to overcome anxiogenic responses to cocaine during daily conditioning sessions. Contrary to typical conditioning protocols, our “pseudo-conditioning” animals had very limited information regarding the lever-pressing outcome because of the random presentation of paired stimuli during conditioning sessions. It has been suggested that anxiogenic effects outweigh the rewarding properties of cocaine when animals have no control over their delivery of intravenous cocaine [101]. Thus, suppressed locomotor activation of our “pseudo-conditioning” animals is likely to be the result of anxiogenic cocaine effects.

It has been proposed that the activity of midbrain DA neurons support and guide learning [17,273-275,334], and the activity of midbrain DA neurons is dependent on “unpredictability” [104]. In this view, the increases in DA levels of animals that have

self-administered cocaine in the presence of the saline-paired stimuli should have been more robust than those tested with cocaine-paired stimuli since the reward is received unexpectedly. However, we observed the opposite results. During the early stage of training, the theory suggests that DA neurons are activated most, yet the cocaine-induced increase in NAcc DA levels was lower in the animals tested with saline-paired stimuli than those tested with cocaine-paired stimuli. Yet, it is not surprising to find different results from electrophysiological studies utilizing conventional rewards and results from the present study utilizing cocaine reinforcement. Thus, the present study, cannot refute the involvement of midbrain DA in "unpredictability" from following reasons: 1) NAcc DA release is reported to be influenced not only by activity of the midbrain, but also influenced by glutamatergic projections from PFC, hippocampus and amygdala [45,155,185,303]. 2) Poor time resolution of microdialysis as compared to real-time measuring of single-unit recording may have resulted in masking the "phasic" DA release which might have occurred in response to cocaine in the presence of the saline-paired stimuli. 3) Neurons recorded in the electrophysiological studies are randomly selected from the midbrain, and thus it is not clear whether these neurons are projecting to the dorsal striatum or to the ventral striatum.

Any external stimuli that are capable of eliciting either approach or escape behavior is described as a "reinforcer" whether it is appetitive or aversive in nature [333]. Stimuli that are capable of eliciting approach or escape behavior must have a biologically significant meaning; otherwise they would not induce such behavior. In this respect, conventional rewards such as food, water, and sex, drugs of abuse as well as aversive

events could be all defined as a reinforcer, all of which are biologically significant to organisms and shown to increase DA in the NAcc [2,72,84,139,337,349,354,355]. In this respect, the mesolimbic dopaminergic system can be said to respond to biologically significant stimuli. For example, a novel stimulus presented to animals, may or may not be determined as biologically significant until associations between stimulus-response and the outcome is learned. The midbrain DA neurons may respond to novel stimuli in such way that animals can learn to make an appropriate behavior when they encounter the stimuli. Alternatively, a putative role of DA in the associative learning as a neural modulator for attentional processes to biologically significant stimuli has been proposed [219,251]. This view is in accordance with the modulatory role of DA on excitability of the medium spiny neuron [140] in the NAcc that serves as a neural interface between limbic and motor system [210].

If responses of the mesolimbic dopaminergic system are modulating associative learning and guiding behavior for successful adaptation to an ever-changing environment, it is plausible to think that successful adaptation can be achieved by sparing the response for new events in the environment. In another words, the mesolimbic DA neurons do not respond to a stimulus that has no biologically significant meaning or to a stimulus that can generate automated performance by learning. If that is the case, it is not surprising to find that the response of the mesolimbic dopaminergic system for the saline-paired stimuli was suppressed during the early stage of learning. The dopaminergic response to the cocaine-paired stimuli was subsequently suppressed as well after Protracted conditioning. Responsiveness of midbrain DA neurons to stimuli is reported to be

decreased or “decay” as learning progresses [145,188]. It was proposed that speed of the “decay” is dependent on saliency of stimuli (see review [273]). For example, activation elicited by mild sensory stimuli decay gradually to baseline during < 100 trials [188]. On the other hand, activation elicited by salient sensory stimuli also decay but still cause measurable activation with > 1,000 trials [147]. Our results lead a speculation that such speed of decay is also applicable to conditioned stimuli, and the speed of decay is dependent on significance of stimuli to biological system. Further research is needed to confirm the notion.

The mesolimbic dopaminergic system has long been known as a neural substrate for mediating reinforcing properties of appetitive stimuli [57], thereby referred to as the “reward circuit.” The same neural system has also been postulated to mediate incentive-motivational properties of cocaine-paired stimuli [106]. Recently however, the notion has been questioned [149]. Our results also showed that conditioned stimuli no longer influenced NAcc DA levels after protracted conditioning. The fact that the NAcc DA is increased by aversive stimuli [2,270,355] and midbrain DA neurons are activated by novel and salient stimuli [147,188], the mesolimbic dopaminergic system is probably not mediating rewarding events per se. If that is the case, the following questions arise. 1) Why do animals lever press to obtain intracranial electrical stimulation (ICS) to brain areas containing DA? 2) Why do pharmacological manipulations of DA alter cocaine self-administration patterns?

ICS is known to be best obtained when electrode is implanted in medial forebrain bundle (MFB) where it contains DA and NE neurons [54]. It was mentioned, however, that electrical stimulation of the MFB is not directly activating the dopaminergic system since conduction velocity estimate of unmyelinated DA axons, which are not easily depolarized by electrical stimulation, does not match with those of MFB neurons [54]. It seems likely that electrical stimulation activates efferent projection from the lateral hypothalamus to the VTA rather than stimulating catecholoaminergic fibers [31]. In fact, a recent *in vivo* voltammetry study failed to observe a rise in NAcc DA release during ICS. In the study, DA increase was observed during early trials but it diminished during continuous stimulation during ICS [113], further suggesting the involvement of the mesolimbic dopaminergic system in an acquisition phase of behavioral learning.

DA antagonists, such as SCH 23390, spiperone [148], and perphenazine [164] are reported to block the reinforcing efficacy of cocaine as assessed by a dose-dependent alteration in cocaine self-administration response rates [352]. Similarly, alterations in cocaine self-administration patterns were observed by destruction of NAcc DA-containing neurons with 6-hydroxydopamine (6-OHDA) [259]. Motoric as well as aversive effects of such dopaminergic manipulation can be inferred. Additionally, this could be explained by DA neurons strengthening the saliency of cocaine rather than influencing the incentive motivational aspects of the drug. Stimuli can gain or loose its saliency by either varying the intensity of a stimulus or by modifying the internal motivational state, thereby influencing the magnitude of DA neuronal response. For example, food rewards are more salient in hungry animals and increase NAcc DA levels

in food-deprived, but not satiated animals [337]. Similarly, aversive stimuli such as foot shock [355] or tail shock [2] increase NAcc DA levels. Very mild non-noxious stimuli, such as air puffs to the hand or drops of saline to the mouth, induce activation on 14% of DA neurons and stimuli conditioned with such non-noxious aversive stimuli activate 11% [208]. Thus, DA may modulate the saliency of stimuli in such a way that manipulations of dopaminergic system may result in alterations of cocaine self-administration patterns.

Considering the modulatory role of the mesolimbic dopaminergic system in the acquisition of associative learning, activation of the system should facilitate associative learning while suppression should delay learning. Some studies support the functional role of the mesolimbic dopaminergic system. For example, when a stimulus (X) was presented with another stimulus (Y) that fully predicts reward, presentation of X alone failed to induce activation in the midbrain DA neurons and failed to induce behavioral responses in animals [322]. Pharmacological manipulations of the NAcc also support the putative role of the mesolimbic dopaminergic system in acquisition of learning. For example, it was reported that acquisition of Pavlovian approach to an appetitive conditioned stimulus was impaired by intra-accumbal infusion of NMDA receptor antagonist [171] and DA receptor antagonist [85]. The DA receptor antagonist effect, however, could be confounded by motoric deficits since intra-accumbal administration of D1/D2 receptor antagonists suppress motor responses [86,202]. Nevertheless, these observations support the role of the mesolimbic dopaminergic system in acquisition of associative learning.

The importance of the mesolimbic dopaminergic system in learning can be further demonstrated in attention-deficit hyperactivity disorder (ADHD) [98]. ADHD is characterized by inattentiveness, impulsivity and hyperactivity [291]. Many of ADHD children are reported to have learning disabilities and poor academic performance [335]. Methylphenidate (Ritalin), a commonly prescribed stimulant drug that blocks DAT, is known to be effective in alleviating these symptoms [291]. Since normal responses observed with dopaminergic agents, methylphenidate and D-amphetamine, is hyperactivity, the response of ADHD children to the dopaminergic agent was referred as "paradoxical" [331]. The "paradoxical" effect of dopaminergic agents was also observed with administration of D-amphetamine to the neonatal rats treated with 6-OHDA [286]. It was postulated that the "paradoxical" therapeutic effects of methylphenidate is a context-dependent enhancement of DA signal amplification [315]. For example, it was found that an effect of food stimulus on food-deprived individuals was augmented by pretreatment with methylphenidate [320]. This observation is in line with *in vitro* electrophysiological findings. DA in the striatal is reported to decreased weak background spontaneous firing rates while it enhances strong input thereby increasing the signal-to-noise ratio of the striatal neurons [140,178]. Moreover, cocaine has been reported to improve accuracy on attentional task at least in cocaine-experienced individuals. Cocaine-induced attentional effects on people without history of cocaine use was not examined due to obvious ethical reasons [165].

Taken together, these observations implicate that impeding mesolimbic dopaminergic activity interferes with acquisition of learning while enhancing the activity facilitates

behavioral learning. This is in accordance with the neural architectural view of the NAcc which suggests the NAcc has an important role for gating information before sending it to the basal ganglia output for goal-oriented behavior [25]. This view is also consistent with a modulatory role of DA in the striatal neurons [140,178]. Further research is required to confirm this notion, since D1-like and D2-like receptor stimulation has been reported to have opposite roles in intracellular signaling cascades [301] and behavioral learning [102].

It is also important to note that in the present study, "pseudo-conditioning" and "cocaine-naïve" groups were included to assess the effects of cues without conditioning and cocaine alone, respectively. However, anxiogenic effects of cocaine and "unpredictability" make it difficult to draw strong conclusions. In follow-up studies, it is important to take such considerations into account. Comparisons should be made between the cocaine-induced NAcc DA increase observed from the present study and from animals that undergo the same number of self-administration sessions without conditioned stimuli. Nevertheless, based on our data and others, it is likely that the mesolimbic dopaminergic system is responsive to general types of stimuli, either novel, appetitive, aversive, or stimuli associated with cocaine. The increased responsiveness is, however, restricted to an early stage of associative learning. The responsiveness of the mesolimbic system to stimuli decays or stimuli loses saliency with repeated exposure. The speed of the decay may depend on significance of stimuli on a biological system. Such phasic activity of the mesolimbic dopaminergic system may be important to facilitate the acquisition of associative learning.

CHAPTER 4. EXPERIMENT 3: EFFECTS OF COCAINE AND SALINE-PAIRED STIMULI ON PFC DA ACROSS DIFFERENT STAGES OF COCAINE-ASSOCIATIVE LEARNING

The purpose of the Experiment 3 was to determine if cocaine-paired stimuli induce changes in mPFC DA levels during early or late stages of cocaine-associative learning. The same experimental procedure was employed with the NAcc experiment except that dialysates were collected from the mPFC. In the following introduction section, I briefly discuss 1) neural architecture and functional role of the mPFC DA, 2) why PFC DA is believed to be an essential component for cocaine addiction, and 3) why the effect of cocaine-paired stimuli on mPFC DA in distinctive stages of cocaine-associative learning is yet be determined. Although other transmitters, particularly acetylcholine, are known to have important roles in cognitive PFC functions [68], it is beyond the scope of this dissertation to include all transmitter systems, thus the discussion will mostly restricted to DA.

4-1. INTRODUCTION

4-1-1. Medial prefrontal cortex and dopamine

In rodents, mPFC is known to receive dopaminergic projections from the VTA (A10), a pathway referred as mesocortical dopaminergic system [54,95,187,249]. A retrograde labeling-immunohistochemical study showed that cell bodies located in the nucleus parabrachialis pigmentosus of the VTA constitute the mesocortical dopaminergic system whereas cell bodies located in the nucleus paranigralis of the VTA form the mesolimbic dopaminergic projection [302]. The mPFC sends back glutamatergic efferents to the

VTA [63] as well as to the NAcc [64], and reported to have intimate functional interconnections with these brain regions [89,170,181,303].

The mPFC in rats is divided into subareas; dorsal mPFC (prelimbic), ventral mPFC (infralimbic) and a superficial layer, anterior cingulate. Both prelimbic and infralimbic are known to receive dense dopaminergic projection while projection to the anterior cingulate is less prominent (see review [308]) but innervated by dopaminergic projections from medial substantia nigra (A 9) and the lateral A 10 region [313]. It has been proposed that the mPFC in rodents is anatomically homologous to mPFC in primates, mainly due to similar reciprocal connectivity with mediodorsal thalamus [226], and functionally analogous to dorsolateral PFC in primates where it is known to play a role in mediating mnemonic, attentional, spatial and associative learning [182]. The subareas of the mPFC are known to be topographically innervated to subterritories of the NAcc; the prelimbic sends projection to the NAcc "core" while the infralimbic and ventral portion of the prelimbic send projection to the NAcc "shell" [19]. The mPFC receives glutamatergic projections from hippocampus, amygdala, mediodorsal thalamus, contralateral mPFC [242] (and review by [131,308]), and inputs from sensory cortices [226]. The mPFC sends efferents to many brain regions, namely cortex areas including posterior cingulate entorhinal and orbital areas, striatum, amygdala, thalamus and brainstem [182]. In addition to the afferent glutamatergic projections, the mPFC is innervated by GABAergic and cholinergic interneurons and cholinergic afferents from the ventral pallidum [32,166]. The glutamatergic, cholinergic and dopaminergic projections are in close apposition to each other, as we see in the striatum, making a

"triad" synaptic arrangement [166] (see review [347]). The "triad" synaptic arrangement provides a suitable place for interaction between these neurotransmitters to occur.

Electron microscopical and immunocytochemical studies showed that the primary target neurons of the mesocortical dopaminergic projections are glutamatergic pyramidal neurons, and to some extent, GABA interneurons [123,313]. DA is known to have modulatory influence on the pyramidal neurons. Generally, iontophoretically applied DA is known to have an inhibitory effect on the pyramidal neurons (see review [308]). When DA is applied with glutamate or acetylcholine however, DA is reported to have opposite effects depending on the concentration. For example, PFC neuronal firing induced by iontophoretic application of acetylcholine was enhanced by a low dose of iontophoretically applied DA. A high dose of DA suppressed both spontaneous and acetylcholine-evoked activity [346]. Whole-cell recordings from the mPFC pyramidal cells also showed that inward currents evoked by NMDA were enhanced by low concentration of DA, whereas higher concentrations of DA suppressed the currents [359]. Although an exact mechanism underlying the dose-dependent effect of DA is not known, since high levels of DA are reported to potentiate glutamate-induced GABA release [78], activation of GABA interneurons in this aspect is speculated. Thus, unlike NAcc DA, which influences excitability of the striatal medium spiny neurons by membrane potential-dependent manner, the mPFC DA modulates the activity of pyramidal cells in a concentration-dependent manner.

4-1-2. Basic function of prefrontal cortex

The PFC is known to be involved in many types of complex behavior, namely response inhibition, recall of order of events, spatial orientation, social and affective behavior, behavioral spontaneity and associative learning [182]. Damage to the PFC is known to affect cognitive performance and memory [13,44,68]. Damage to the PFC is also reported to result in impulsive personality. For example, patients with damage in the PFC have a tendency to choose high immediate gain with larger future loss over lower immediate gain but a smaller future loss in a "gambling task" [13]. Such impulsive behavior has also been observed in animals with mPFC lesions. Weissenborn and associates (1997) showed that when rats were trained to lever press to obtain a stimulus associated with cocaine via a second-order schedule, omission of the stimulus resulted in suppression of the lever pressing in control animals. Rats with mPFC lesions exhibited impaired extinction of the lever responses [330]. Impaired extinction of a conditioned fear response was also observed in rats with mPFC lesions [247]. However, when rats were given a choice between a larger, but delayed food reinforcer and a smaller, but immediate reinforcer, animals with mPFC lesions failed to show impulsivity [53]. These findings suggest that the impaired extinction in the animals may not simply due to impulsivity.

It was found that animals with mPFC lesions have no difficulty in the acquisition of fear conditioning [211,247] but exhibit prominent impairment in extinction of the conditioned fear response 24 hours after the extinction training [247]. This has led to a speculation that mPFC lesions cause a deficit in the re-wiring of stimulus-response memory already formed. A recent single-unit recording study by Milad and Quirk showed robust activity

in the mPFC during the recall trial of extinction but not during extinction training. In fact, animals that exhibited more neuronal activity in the mPFC showed more successful extinction behavior. They also found that electrical stimulation of the mPFC during the extinction recall trial enhanced extinction behavior [203]. These observations suggest mPFC dysfunction may impair post-training memory consolidation processes.

Behavioral deficits manifested with PFC damage have also been described as "stimulus-bound" or "prepotent responses," where behavior is known to be largely driven by previously learned stimuli. The result is fixated behavior, or an inability to override such behavior [204]. Such fixated behavior has been described in humans with PFC damage using the Wisconsin-Card Sort Test (WCST). In WCST, subjects are instructed to sort a deck of cards containing different shapes, colors and numbers without being told the rule for sorting. Once the subjects figured out the rule and learned to respond appropriately, the experimenter changes the rule without telling the subjects. Normal subjects are known to be able to successfully switch their strategy according to the new rule. On the other hand, patients with PFC damage have difficulty switching their strategy when the rule was changed, even though they quickly learned the first rule [55]. Thus, PFC deficits are suggested to result in inability to re-wire previously learned responses. As a result, their behavior may be strongly driven by previously learned stimuli-response. This view is in accordance with the aforementioned findings from experimental animals, and may describe why PFC lesion results in impairment in extinction [247,330] but not impulsivity [53].

4-1-3. Functional role of dopamine in the prefrontal cortex

It has been suggested that there is an inverted U-shape range of optimal cortical D1-like receptor stimulation referred as “optimal narrow window” for proper cognitive function [291,358]. Thus, too much or too little DA in the PFC is known to impair cognitive processes. For example, supranormal stimulation of PFC with D1 agonist was shown to impair spatial memory in rats, and this effect was blocked by D1 antagonist treatment [358]. Infusion of a D1 antagonist itself also produced impairment in the spatial memory [276]. D1 antagonist treatment was also shown to impair cognitive function in monkeys, while pretreatment with the same dopaminergic agents restored cognitive deficits induced by acute stress [10]. It was also reported that hypo-dopaminergic turnover in the PFC induced by chronic stress impaired spatial memory in rats, which was ameliorated by intra-PFC infusion of D1 agonist [209]. Interestingly, intra-mPFC infusion of a D1 agonist was shown to improve performance only in rats performing at a low level of accuracy, while D1 antagonist treatment impaired performance only in animals performing at a high level of accuracy [128]. These findings suggest that the effects of such dopaminergic agents can be dependent on preexisting individual differences. Nevertheless, these observations implicate that both hyper- and hypo-dopaminergic transmission in the PFC result in impaired cognitive function.

As it was mentioned, DA is known to have a modulatory influence on the activity of mPFC pyramidal neurons in concentration-dependent manner. High doses of DA are reported to suppress the neural activity evoked by glutamate or acetylcholine, while low doses enhance activity [346,359]. Tzschentke proposed a “gating” effect of DA on the

excitatory inputs as a neural basis for the effect of “optimal narrow window” of DA levels on optimum cognitive function. According to this hypothesis, too much of DA would shut the “gate” for excitatory inputs to pyramidal cells while too low of DA would result in “interferences” induced by different excitatory inputs reaching simultaneously [308]. Currently, however, the exact neural basis of how such levels of DA influence cognitive function is not known.

4-1-4. Medial prefrontal cortex and cocaine

Jentsch and Taylor suggested an important role of the PFC in the etiology of drug addiction. It was postulated that dysfunction of the PFC results in impaired inhibitory control over inappropriate behavior leading to compulsive drug-seeking and drug-taking [161]. However, this notion has not been fully investigated.

Nevertheless, a large amount of literature exists concerning the role of the mesocortical dopaminergic system in cocaine addiction. Functional imaging studies in cocaine-dependent humans have shown that cocaine resulted in regional activation in the PFC [40]. In experimental animals, it has been reported that cocaine increases extracellular DA levels in the mPFC [150,294] and is also directly self-administered into the mPFC [120,122]. In the study, substitution of cocaine to lidocaine did not maintain the intracranial self-administration, suggesting that the rewarding effects of cocaine in the mPFC were not due to a local anesthetic effect [120]. Lesions of the PFC are reported to result in attenuation of the intracranial cocaine self-administration [122], cocaine conditioned place preference (CPP) [309], and alterations in intravenous cocaine self-

administration response patterns [197]. These studies implicate the involvement of PFC in cocaine addiction.

4-1-5. Medial prefrontal cortex and cocaine-associated stimuli

Emerging evidence suggests that the PFC may also be involved in the conditioning effects of cocaine. Functional imaging studies in cocaine-experienced individuals have shown that cocaine-paired stimuli induce regional activation in the PFC during cue-induced craving [129,190]. In experimental animals, exposure to a cocaine-paired environment has been shown to increase mPFC *c-fos* expression, [266,267], increase locomotor activity and reinstate cocaine-seeking behavior [65,214]. Systemic administration of DA D1 antagonist before the reinstatement test session was reported to suppress both increased mPFC *c-fos* expression and reinstatement behavior [65]. These observations implicate involvement of mPFC in incentive motivational properties of cocaine-paired stimuli. The role of mPFC DA in this respect is yet to be determined.

In the NAcc experiment, we showed the influence of cocaine-paired stimuli on NAcc DA was dependent on the stage of associative learning. Briefly, cocaine-paired stimuli influenced NAcc dopaminergic response when animals were in an early phase of training, but the effect was blunted in a late phase of training. Since both mPFC and NAcc receive dopaminergic projections from the VTA (A 10) [54,95,187,249], does the response of mPFC DA show changes similar to the NAcc? Or, since the mesocortical and mesolimbic dopaminergic projections arise as distinctive population of neurons in the VTA [302], does the mPFC show distinctive response patterns to cocaine-paired stimuli?

Several findings suggest that mPFC DA responds differently from NAcc DA. An *in vivo* microdialysis study showed that mPFC DA responded to conventional rewards persistently even after repeated exposure of the rewards while response of NAcc DA was blunted by the pre-exposure [12]. An *in vivo* voltammetry study also showed increased DA release in the mPFC evoked by repeated electrical stimulation of the VTA while response of the NAcc decreased [112]. Moreover, neuronal activation induced by cocaine-paired stimuli was found in the PFC, but not in the NAcc in animals that had well-learned discriminatory behavioral responses to cocaine-paired stimuli [65]. These observations suggest an important role of the mPFC in reward-related associative learning particularly after habituation to the reward occurred. Thus, we hypothesized that unlike NAcc DA, mPFC DA would be responsive to cocaine-paired stimuli even after protracted conditioning. In order to determine whether the responsiveness of the mesocortical dopaminergic system to cocaine-paired stimuli changes across different stages of cocaine-associative learning, DA overflow in the mPFC in response to cocaine-paired stimuli was measured from rats after Limited or Protracted self-administration conditioning.

4-2. METHODS

Subjects: Male albino Sprague-Dawley rats (Animal Resource Center, Austin, TX) weighing approximately 250 – 300 g at the beginning of the experiments were used (N = 46). The animals were group-housed and maintained on a 12 hr. reversed light/dark cycle (light on 7:00 p.m. to 7:00 a.m.). They were handled daily as described in Experiment 1. Food and water were available *ad libitum* in the home cage except during food training.

Apparatus: Food training, self-administration sessions, and *in vivo* microdialysis test sessions were conducted in identical one-lever operant chambers described in Experiment 1.

Surgery: Prior to surgery, animals were trained to lever press for food on a fixed ratio 1 (FR1) schedule of reinforcement as described in experiment 1. After the completion of the food training, animals were implanted with a chronic silastic intravenous jugular catheter (see Methods, Experiment 1). Those animals were also stereotaxically implanted with an unilateral guide cannula (21 ga) aimed to 3.55 mm above the mPFC (AP: + 3.8 mm; ML: ± 0.6 mm; DV: -1.5 mm) according to the atlas of Paxinos and Watson (1997) [232]. Guide implantation in the left versus the right mPFC was counterbalanced across animals. Animals underwent a minimum of a one-week recovery prior to the beginning of the experiments. Catheter patency was maintained by infusing one U/ml streptokinase and 30 U/ml heparin mixed in 0.1 cc of isotonic saline as described in experiment 1.

Cocaine/Saline Conditioning Self-administration Sessions: Over the course of conditioning sessions animals had alternating days of cocaine and saline availability during one-hour daily self-administration sessions (e.g., 6 days of cocaine and 6 days of saline sessions = Limited training and 20 days of cocaine and 20 days of saline sessions = Protracted training) as described in Experiment 2. Briefly, after the first 30 min of habituation to the chamber, the olfactory (cinnamon or rose oil-based scents) and visual cues (black or white felt “walls”) paired with either cocaine or saline was introduced into

the operant chamber, the lever was protruded, and cocaine or saline then became available for 30 min. During these sessions, each lever press resulted in the delivery of 0.5 mg/kg/0.1 cc cocaine hydrochloride mixed in isotonic saline (cocaine days), or saline alone (0.1 cc) (saline days), infused over 6 seconds (verification of the dose used is in the method section of experiment 1). After each infusion, there was a 20-sec "time-out" period, during which time the lever was retracted, the stimulus light was off and no infusions were available. As described in Experiment 2, "pseudo-conditioning" and "cocaine-naïve" groups were included to assess the effects of cues without conditioning and cocaine alone, respectively. The "pseudo-conditioning" group underwent the same number of conditioning sessions as the Limited training groups (i.e., total of 12 days conditioning sessions) but the cues were presented randomly during the self-administration sessions. In this group, the visual and the olfactory cues were randomly assigned to each day of the 12 daily conditioning sessions. The "cocaine naïve" animals were also placed into the operant chamber for the same number of days, but they were removed from the chamber after the 30-min habituation period. Animals failed to reach a criterion response (mean responding ≤ 1) for cocaine were excluded from the experiment as described in Experiment 2.

In Vitro Recovery Calibration: Prior to probe recovery, all probes were calibrated as the same procedure as in experiment 2 (see Methods, Experiment 2). Briefly, ten-min samples from each probe were collected and assayed by high performance liquid chromatography (HPLC) with electrochemical detection (HPLC-EC). Probe recovery were calculated by comparing the peak heights of samples to those from a standard (5 nM

DA). The recovery of probes used in the experiment (expressed as mean ± SEM) was 16.12 ± 0.43 %.

Microdialysis probe implantation: After completion of all self-administration sessions, animals were briefly anesthetized with Methohexital sodium (Brevital®, approximately 6 mg/i.v.) and implanted with a microdialysis probe through the previously implanted guide cannula. The microdialysis probe were connected to a 1.0 ml gastight Hamilton 1000 series syringe mounted on a syringe pump (Razel®, Model A), and freshly prepared Ringer's solution was pumped through the probe. Animals implanted with the probe remained in a holding chamber overnight with the syringe pump speed set at 0.261 μl/min. Bedding, food, and water were available in the holding chamber. Thirty min prior to the test session, the pump speed was changed to 1.63 μl/min.

Test Day conditions: At least 12 hours after the probe implantation, animals were tested for locomotor and dopaminergic responses before and after a challenge dose of self-administered infusion of either cocaine (3.0 mg/kg) or saline (0.1 cc) as described in Experiment 2. Briefly, animals were placed in the darkened operant chamber as during the training sessions for the first 30 min. After the 30 min., the house light illuminated, the olfactory and the visual cues were introduced into the operant chamber, and the lever extended into the chamber. For animals tested with cocaine-paired stimuli ("CS"), the olfactory and visual cues that had been conditioned with cocaine were present. For animals tested with saline-paired stimuli ("SS"), testing was conducted with the cues paired with saline-training days. For "pseudo conditioning" group ("RS"), testing was

conducted with one pair of the olfactory and visual cues randomly presented during conditioning sessions. After the animal pressed the lever, either 0.1 cc of 3.0 mg/kg cocaine mixed in saline or saline was intravenously delivered over 6 seconds. Brain dialysate and locomotor activity data were collected in 10-min sample bins over the course of the one-hour session; 3 baselines during the habituation periods and 3 post self-infusion samples. The average of baseline DA levels was defined as 100% and individual samples were represented relative to 100%. Locomotor activity was recorded via computer assessment of photobeam interruptions inside the operant chamber.

Assay of dialysate: The dialysates were analyzed for DA concentrations using HPLC equipped either with a) ESA Catecholomine HR80 reverse-phase column or Rainin microsorb-MV C-18 RP column, ESA Model 5200A Coulochem II Detector, a Model 5020 Guard Cell and a Model 5014B Choulmetric Analytical Cell with the detection limit for DA as 0.29 pg/sample or b) Shizeido capcell C-18 narrow bore column, ESA Model 5200 A Coulochem II Detector, a Model 5020 Guard Cell and a Model 5041 Amperometric Analytical Cell with the detection limit for DA as 0.09 pg/sample. For the HPLC set up and mobile phase composition, see Method section of Experiment 2. Data was collected and analyzed using an ESA Model 500 Data station.

Histological analysis: After the experiment, animals were euthanized by administering an overdose of sodium pentobarbital (Nembutal) and brains were removed and stored in 10 % formaldehyde solution with 30 % sucrose. The probe placements of each animal were verified using the atlas of Paxinos and Watson (1997) [232] from coronal sections

(48 µm) stained with cresyl violet. Animals in which active membrane region outside of the mPFC were excluded from further analysis. Fig. 12-A & B shows the probe traces of the coronal sections. Fig. 13 shows a dialysis probe trace in the coronal section from a representative animal.

Data analysis: Dialysate DA in the Limited training and Protracted training groups were analyzed separately by two-way ANOVAs (Condition or Group x Time) with repeated measures on the Time factor. When there were main effects or interactions by the overall analysis, *Post hoc* analyses (Fishers LSD) were used to detect significant differences at specific time points.

4-3. RESULTS

Lever responses during the cocaine self-administration sessions: The mean number of lever responses (mean ± SEM) for cocaine from animals that underwent the limited training were 2.40 ± 0.38, 4.53 ± 0.70, and 3.93 ± 0.70 for "pseudo-conditioning ("RS"), animals tested with cocaine-paired stimuli ("CS"), and animals tested with saline-paired stimuli ("SS"), respectively. The corresponding cocaine amounts for the mean lever responses were 1.2 ± 0.19 mg/kg, 2.27 ± 0.35 mg/kg, and 1.97 ± 0.35 mg/kg for "RS," "CS" and "SS," respectively. A one-way ANOVA showed that the lever responses for cocaine were comparable between groups [F (2,23) = 1.736; NS]. The mean number of lever responses (mean ± SEM) for cocaine from animals that underwent the protracted trainings was 6.09 ± 0.82 and 7.66 ± 0.96 for "CS" and "SS," respectively. The corresponding cocaine amounts for the mean lever responses were 3.05 ± 0.41 mg/kg for

"CS" and 3.83 ± 0.48 mg/kg for "SS." A t-test showed no significance differences between them [t (14) = -1.2411; NS].

Effect of paired cues on PFC DA levels from the Limited training animals: Fig. 14-A shows the DA response at the test session from the following groups that self-administered cocaine. **1)** Animals that received a self-injection of cocaine (3.0 mg/kg) in the presence of cocaine stimuli ("Cocaine + CS," n = 5), **2)** animals that received a self-injection of cocaine in the presence of saline stimuli ("Cocaine + SS," n = 5), **3)** "pseudo-conditioning" group that received a self-injection of cocaine in the presence of randomly conditioned stimuli ("Cocaine + RS," n = 5), and **4)** "Acute" animals that received a self-injection of cocaine in the first time at the test session ("Acute," n = 4). The mean (mean ± SEM baseline DA concentration were 0.18 ± 0.01 nM, 0.16 ± 0.02 nM, 0.24 ± 0.03 nM, 0.12 ± 0.03 nM for "Cocaine + RS," "Cocaine + CS," "Cocaine + SS," and "Acute," respectively. A one-way ANOVA showed their baseline DA concentrations were significantly different between groups [F (3, 15) = 4.5733; $p < 0.05$]. *Post hoc* tests revealed that the baseline DA concentrations of "Cocaine + SS" were significantly higher than that of "Cocaine + CS" and "Acute," $p < 0.05$ and $p < 0.01$, respectively.

DA levels significantly increased after the cocaine infusion in all groups ($p < 0.05$ for "Cocaine + SS," $p < 0.01$ for "Cocaine + CS," "Cocaine + RS" and "Acute," Fig. 14-A). A two-way ANOVA (Group X Time) with repeated measures revealed significant effects of Group [F (3,15) = 15.2448; $p<0.01$], Time [F (5,75) = 52.7991; $p < 0.01$] and a Group by Time interaction effect [F (15,75) = 7.7997; $p < 0.01$]. *Post hoc* tests revealed that the

cocaine-induced increase in DA levels of "Cocaine + RS" and "Acute" groups were significantly higher than "Cocaine + CS" and "Cocaine + SS" across all three post-infusion time points. The cocaine-induced increase in DA levels were also higher in "Acute" animals than that seen in "Cocaine + RS" at the first 10-min and the last 10-min sampling points ($p < 0.01$). DA levels of "Cocaine + CS" was also significantly higher than those of "Cocaine + SS" during the last 10-min interval after the cocaine infusion ($p < 0.05$).

Fig. 15-A shows the DA response from animals that underwent the limited conditioning and received a self-injection of saline at the test session with either cocaine-paired stimuli ("Saline + CS," n = 5) or with saline stimuli ("Saline + SS," n = 6). The mean (mean ± SEM) baseline DA concentration from these animals was 0.2 ± 0.02 nM for "Saline + CS" and 0.14 ± 0.02 nM for "Saline + SS." A t-test showed that there was no significant difference in their baseline DA levels between groups [t (9) = 1.9688; NS]. When animals received a self-infusion of saline, DA levels did not show significant changes between pre- and post-infusion or between groups. A two-way ANOVA (Group X Time) with repeated measures showed no significant effects of Group [F (1,9) = 0.5056; NS], Time [F (5,45) = 2.2443; NS] and no interaction effects [F (5,45) = 1.5604; NS].

Effect of paired cues on locomotor activity from the Limited training animals: Fig. 14-B shows locomotor activity from animals that underwent the limited training and self-administered cocaine at the test day ("Cocaine + CS," "Cocaine + SS," "Cocaine + RS" and "Acute"). Following the self-injection of cocaine, locomotor activity increased

significantly in all groups ($p < 0.01$ for "Cocaine + CS," "Cocaine + SS" and "Cocaine + RS"; $p < 0.05$ for "Acute"). A two-way ANOVA (Group X Time) with repeated measures revealed no significant effects of Group [$F(3,15) = 0.1566$; NS], but a significant Time effect [$F(5,75) = 22.5892$; $p < 0.01$], and no significant Group X Time interaction effects [$F(15,75) = 1.0088$; NS]. *Post hoc* tests revealed that the cocaine-induced increase in the locomotor hyperactivity was significantly greater in "Cocaine + CS" than "Cocaine + RS" and "Acute" ($p < 0.01$).

When animals received a self-infusion of saline, their locomotor activity was comparable between groups ('Saline + CS' and 'Saline + SS') (Fig. 15-B). A two-way ANOVA (Group X Time) with repeated measures showed a no significant Group [$F(1,9) = 1.0598$; NS], or interaction effects [$F(5,45) = 0.7833$; NS], but a significant effect of Time [$F(5,45) = 4.2162$; $p < 0.01$], Both groups had slightly greater locomotor activation at the first baseline-sampling bin. *Post hoc* tests revealed that there was no significant difference between groups across all time points.

Effect of paired cues on PFC DA levels from the Protracted training: Fig. 16-A shows the DA response from animals that underwent the protracted training and received a self-injection of cocaine (3.0 mg/kg) at the test session in the presence of either cocaine-paired stimuli ("Cocaine + CS," $n = 5$) or saline-paired stimuli ("Cocaine + SS," $n = 4$). The mean (mean ± SEM) baseline concentration of their DA levels was 0.116 ± 0.0367 nM and 0.175 ± 0.0473 nM in "CS" and "SS," respectively. A t-test showed no significant differences in baseline DA levels between groups [$t(7) = -1.0031$; NS].

Cocaine infusion induced a significant DA increase only in animals tested with cocaine-paired stimuli (Fig. 16-A). A two-way ANOVA (Group X Time) with repeated measures revealed a no significant differences between Groups [$F(1,7) = 1.595$; NS], and no significant Group X Time interaction effects [$F(5,35) = 1.587$; NS], but a significant effect of Time [$F(5,35) = 8.4384$; $p < 0.01$]. *Post hoc* tests revealed that the cocaine-induced increase in DA levels of "Cocaine + CS" were significantly higher than those observed in "Cocaine + SS" at the first 10-min after the cocaine infusion ($p < 0.01$).

Fig. 17-A shows the DA response from animals that underwent the protracted conditioning and received a self-injection of saline in the presence of either cocaine-paired stimuli ("Saline + CS," n = 4) or saline-paired stimuli ("Saline + SS," n = 3). The mean (mean ± SEM) baseline concentration of their DA levels was 0.10 ± 0.02 nM and 0.16 ± 0.06 nM in "Saline + CS" and "Saline + SS," respectively. A t-test showed no significant differences in baseline DA levels between groups [$t(5) = -0.9839$; NS].

Saline infusion resulted in depression of the DA levels only in the animals tested with the saline-paired stimuli (Fig. 17-A). A two-way ANOVA (Group X Time) with repeated measures showed no effect of Group [$F(1,5) = 3.3011$; NS], and Group X Time interaction [$F(5,25) = 2.0929$; NS], but a significant effect of Time [$F(5,25) = 2.7421$; $p < 0.05$]. *Post hoc* tests revealed that during the first10-min post-saline interval, DA levels in animals tested with cocaine-paired stimuli were significantly lower than DA levels of animals tested with saline-paired stimuli ($p < 0.05$).

Effect of paired cues on locomotor activity from the Protracted training animals:

Fig. 16-B shows the effect of self-administered cocaine on locomotor activity at the test day from animals that underwent the protracted training. Following the self-injection of cocaine, locomotor activity significantly increased only in animals tested with cocaine-paired stimuli ("Cocaine + CS") while cocaine-induced increase in their locomotor activity was subtle in animals tested with saline-paired stimuli ("Cocaine + SS"). A two-way ANOVA (Group X Time) with repeated measures revealed no significant effects of Group [$F(1,7) = 0.598$; NS], and no significant Group X Time interaction [$F(5,35) = 1.0757$; NS], but a significant effect of Time [$F(5,35) = 4.7786$; $p < 0.01$]. *Post hoc* tests revealed the cocaine-induced locomotor hyperactivity in animals tested with cocaine-paired stimuli was significantly higher than the cocaine-induced increase in the animals tested with saline-paired stimuli during the first 10-min post-cocaine interval ($p < 0.05$).

When animals received a self-infusion of saline ("Saline + CS" and "Saline + SS"), there was no significant difference in their locomotor activity between groups (Fig. 17-B). A two-way ANOVA (Group X Time) with repeated measures showed no effects of Group [$F(1,5) = 0.4499$; NS], but a significant effect of Time [$F(5,25) = 6.1459$; $p<0.01$], and no significant Group by Time interaction effects [$F(5,25) = 0.6583$; NS]. In both groups, locomotor activity after the saline infusion was significantly depressed from basal levels.

Fig. 12

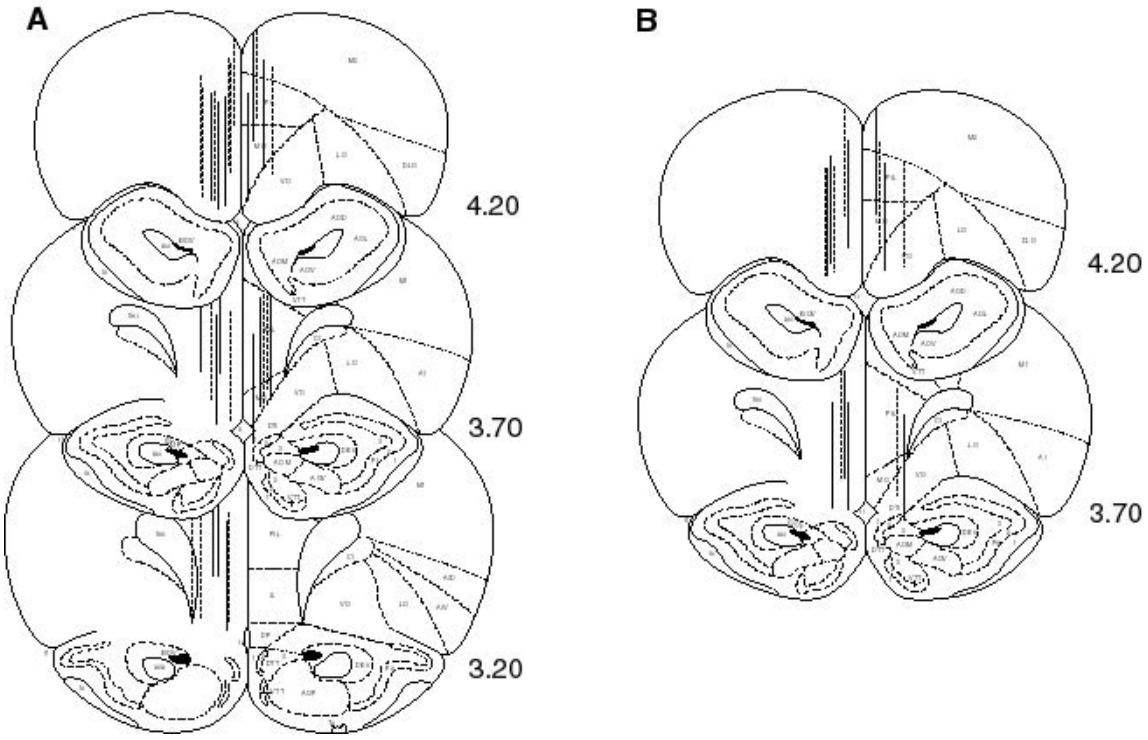

Fig. 12. Schematic representation of the active dialysis probe membrane region in the mPFC of animals that completed the dialysis experiment with **(A)** the limited conditioning (N = 30) or **(B)** the protracted conditioning (N = 16). Numbers depicted next to each brain slice indicate the mm anterior to bregma. The diagram was drawn with the assistance of the atlas of Paxinos and Watson (1997) [232]. Solid lines indicate animals that were tested with a self-infusion of cocaine. Dotted lines indicate animals that were tested with a self-infusion of saline.

Fig. 13

Fig. 13. A dialysis probe trace in the mPFC from a coronal section (48 μm) of a representative animal. Width is 976 % and Height is 988 % from the actual size.

Fig. 14

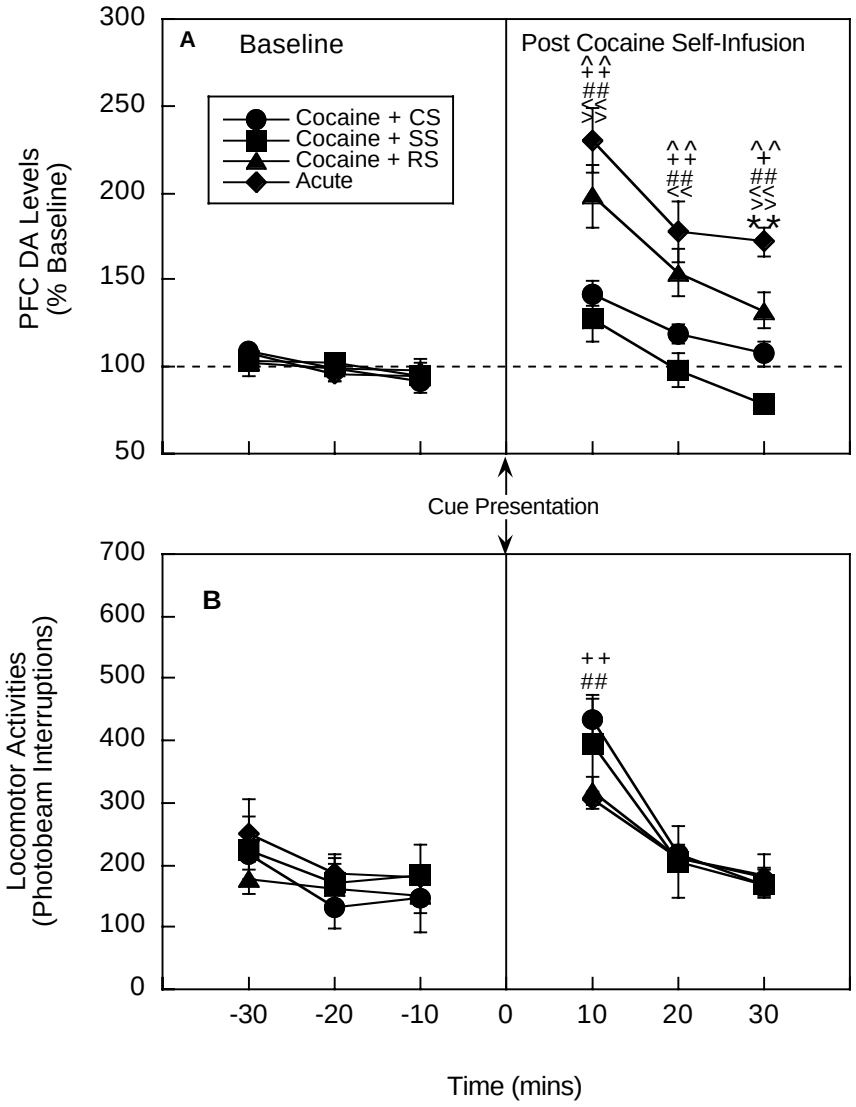

Fig. 14. Effect of paired cues and self-infused cocaine on **(A)** PFC DA levels and **(B)** behavioral responses from Limited training animals (● = Cocaine + CS; ■ = Cocaine + SS; ▲ = Cocaine + RS; ◆ = Acute). **(A)** Cocaine + RS and Acute had a greater DA increase than Cocaine + CS and Cocaine + SS in all post-infusion time points (++, +, and ^^ = $p < 0.01$ and $p < 0.05$ in Cocaine + CS and Cocaine + SS versus Cocaine + RS, respectively; ## and << = $p < 0.01$ in Cocaine + CS and Cocaine + SS versus Acute, respectively). The increase was also greater in Acute than in Cocaine + RS at the first and the last 10-min post-infusion (>> = $p < 0.01$). The increase was also greater in Cocaine + CS than Cocaine + SS during the last 10-min post-infusion (**, $p < 0.01$). **(B)** Cocaine significantly increased locomotor activity in Cocaine + CS, Cocaine + SS, and Cocaine + RS, ($p < 0.01$). The increase was significantly higher in Cocaine + CS than Cocaine + RS (++, $p < 0.01$) or Acute (##, $p < 0.01$) at the first 10-min post-infusion.

Fig. 15

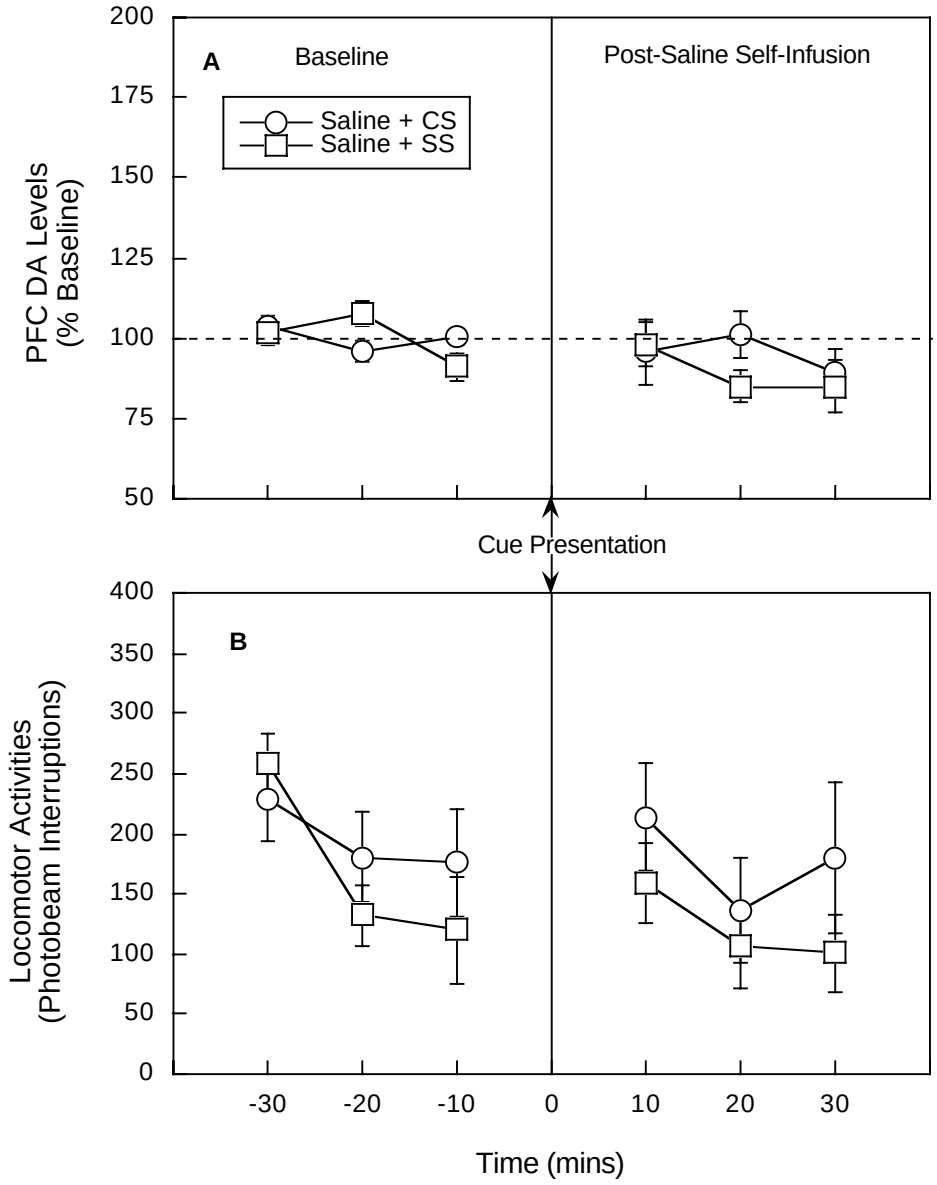

Fig. 15. Effect of paired cues with a self-infused saline on **(A)** PFC DA levels and **(B)** behavioral responses from the limited training animals (○ = "Saline + CS"; □ = "Saline + SS"). **(A)** A self-administered saline did not significantly change DA levels. **(B)** There was no significant effect of the paired cues on their locomotor activity.

Fig. 16

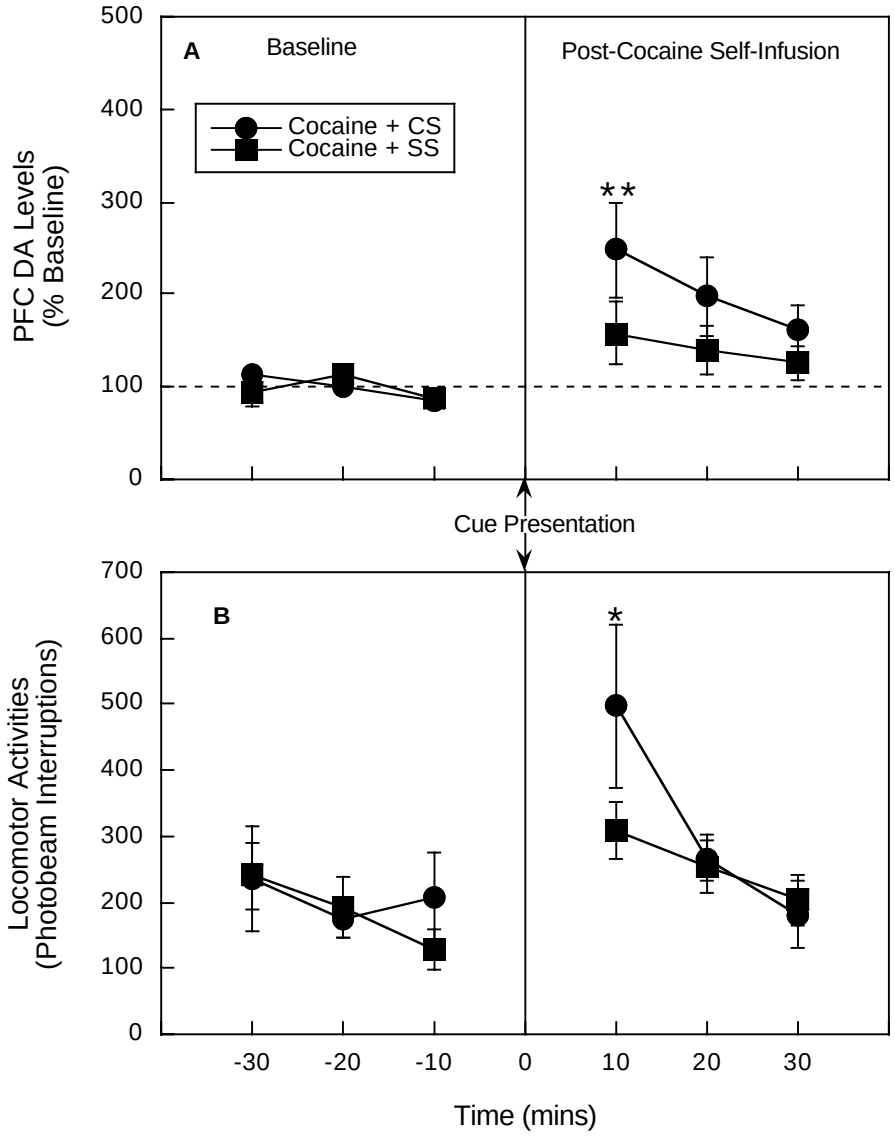

Fig. 16. Effect of paired cues with a self-infused cocaine on **(A)** PFC DA levels and **(B)** behavioral responses from the protracted training animals (● = "Cocaine + CS"; ■ = "Cocaine + SS"). **(A)** Cocaine infusion induced a significant DA increase only in "Cocaine + CS." The increase in "Cocaine + CS" was significantly higher than that seen in "Cocaine + SS" at the first 10-min post-cocaine infusion (**, $p < 0.01$). **(B)** Cocaine induced significant increase in locomotor activity only in "Cocaine + CS." The increase in "Cocaine + CS" was significantly higher than that seen in "Cocaine + SS" during the first 10-min post-cocaine interval (*, $p < 0.05$).

Fig. 17

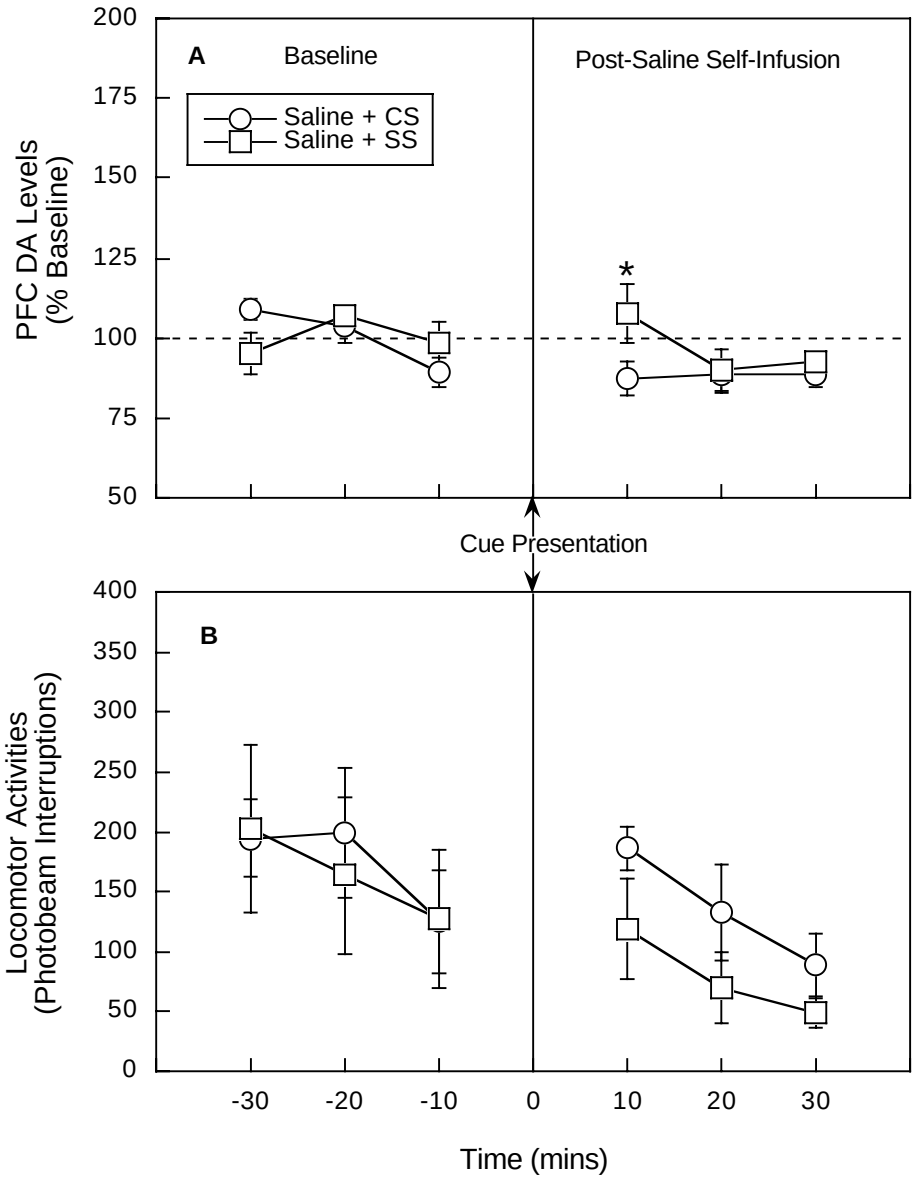

Fig. 17. Effect of paired cues with a self-infused saline on **(A)** PFC DA levels and **(B)** behavioral responses from the protracted training animals (○ = "Saline + CS"; □ = "Saline + SS"). **(A)** DA levels of "Saline + CS" were significantly lower than that seen in "Saline + SS" during the first 10-min post-saline interval (*, $p < 0.05$). **(B)** In both groups, locomotor activity after the saline infusion significantly depressed from baseline, but there was no significant difference between groups.

4-4. DISCUSSION

A major finding of the present study is that the effects of cocaine and cocaine-conditioned stimuli became prominent with the protracted conditioning. Cocaine-paired stimuli enhanced cocaine-induced increases in mPFC DA levels in animals that underwent Protracted conditioning (Fig. 16-A). This effect was subtle in animals with Limited conditioning (Fig.14-A). Similarly, when animals self-administered saline at the test session, cocaine-paired stimuli influenced mPFC DA levels only in animals that underwent the protracted conditioning (Fig. 17-A). Therefore, unlike the accumbal dopaminergic system, the cortical dopaminergic response to cocaine-paired stimuli is enhanced by prolonged conditioning.

Few studies have examined the effects of cocaine-paired stimuli on the PFC utilizing a self-administration paradigm. Among them, few have found effects of cocaine-paired stimuli on the mPFC. For example, it was reported that cocaine-paired stimuli induced increase in *c-fos* expression in the mPFC of rodents [65,214]. In these studies, animals were tested after they had well-established discriminatory lever responses to cocaine. As a result, the number of self-administration training sessions were similar to the length of conditioning in our Protracted training groups. Thus, prolonged conditioning may also be the key determinant for the PFC neuronal activation induced by cocaine-paired stimuli. Our data implicate involvement of DA in this aspect. It is, however, not clear that the neural activation is caused by the increased DA levels in the mPFC since activity of mPFC pyramidal neurons are reported to be suppressed by high concentration of DA

[346,359]. Moreover, *c-fos* expression can be induced not only by DA but can also be induced via variety of stimuli and synaptic stimulation that activate adenylate cyclase signaling cascades [266]. Thus, increased *c-fos* expression and DA levels could be resulted from independent events. Immediate early gene *c-fos* expression was thought to be as a marker of neuronal activation; similar to 2-deoxyglucose that measures neuronal metabolic activity [267]. It has been proposed, however, that expression of *c-fos* trigger neural changes that underlies experience-dependent neural plasticity. Thus, *c-fos* expression in the brain is related to the learning of new experience and not simply linked to neural metabolic activity [66]. Accordingly, it was found that the expression of *c-fos* mRNA of young chicks was subjected to experience-dependent stimulation that can be induced without elevation of metabolic activity [9]. Although the relationship between enhanced DA release found in our study and increased *c-fos* expression found by others can not be elucidated, our results suggest that after prolonged conditioning, cocaine-paired stimuli become capable of influencing DA levels in the mPFC. This result is in accordance with findings from the immunohistochemical studies. These findings may be predictive of factors in human cocaine addiction since it has been suggested that the distribution of the neuronal activation induced by cocaine-paired stimuli in rodents [65] closely paralleled data from brain-imaging studies in humans [129,190]. In addition, the afferent dopaminergic projections to the mPFC in rodents are densely innervated as those seen in primates [21].

Similar to the PFC DA response, Protracted conditioning enhanced the locomotor response to cocaine in the presence of cocaine-paired stimuli (Fig.16-B). This finding

was identical to what we found in animals implanted with the NAcc probes (Fig. 9-B, Experiment 2). Briefly, cocaine-paired stimuli augmented cocaine-induced locomotor hyperactivity in the animals that underwent Protracted training. No effects of paired stimuli were observed in animals that underwent Limited conditioning. Since cocaine-paired stimuli also augmented mPFC DA levels in the animals, one might speculate that the greater increase in PFC DA levels may have resulted in the locomotor hyperactivation or vice versa. However, data from our animals conditioned with random cues ("pseudo-conditioning") disputes that notion. The "pseudo-conditioning" animals had greater mPFC DA increases after a cocaine infusion than other conditioned groups while their corresponding locomotor activity levels was lower (Fig.14-A & B). Using single-unit recording techniques, Chang et al., have also showed that mPFC neuronal activity is dissociable from their movements [60]. Therefore, it is unlikely that the higher mPFC DA levels underlie locomotor hyperactivity in our animals. Based on the pseudo-conditioning findings, mechanisms underlying enhanced locomotor responses are likely to be independent of mPFC DA levels.

Our results indicate that chronic discriminative conditioning enhanced mPFC DA responses to cocaine-paired stimuli. If chronic conditioning is the determinant for PFC DA responsiveness, the impact of cocaine-paired stimuli on our "pseudo-conditioning" and "acute" animals should be minimal. Unexpectedly, as it was observed in the NAcc animals, the cocaine-induced increase in DA levels was greater in the "pseudo-conditioning" and "acute" animals. It could be that the robust DA increase in the mPFC of the "pseudo-conditioning" and "acute" animal was due to phasic learning signals. Yet,

it was reported that excitotoxic lesions of the mPFC have no effect on acquisition of Pavlovian approach behavior in contrast to the marked deficit observed in animals with lesions of the NAcc "core" ([258] review). Alternatively, as it suggested in the Discussion section of Experiment 2, the hyperdopaminergic response of the "pseudo-conditioning" and "acute" animals may be due to augmentation of DA overflow induced by anxiogenic-like stress response to intravenous cocaine. Stressful events have been reported to suppress locomotor activity in rodents [81,348,353]. The "pseudo-conditioning" and "acute" animals exhibited lower activity levels after cocaine infusion as compared to other conditioned groups. Stress has also been reported to increase DA levels more so in the mPFC than in the NAcc [2]. The percentage increase in DA levels from "pseudo-conditioning" and "acute" animals in the present study was, however, higher in the NAcc than in the mPFC. If stress increases DA levels more so in the mPFC than in the NAcc, would the greater increase be seen in the NAcc tested animals? Possible explanations could include the pharmacological action of cocaine on DAT in conjunction with stress-induced DA increases. Since DAT blockade is known to have smaller effects on extracellular DA levels in the mPFC as compared to the NAcc (review by [308]), it is not surprising to see such differences in the findings. Nevertheless, as previously suggested in Experiment 2, it is important to take "anxiogenic" properties of cocaine into consideration in future experiments.

It has been proposed that PFC dysfunction results in impaired inhibitory control over inappropriate behavior, leading to compulsive drug-seeking and drug-taking behavior [161]. The theory arose from observations in which patients with PFC damage and

animals with mPFC lesion manifest deficits in response inhibition and deficits revising previously learned stimulus-response behaviors [13l,247,330]. In fact, it was shown that animals with mPFC lesions exhibited impaired extinction of lever presses previously associated with cocaine, even after omission of a secondary reinforcer [330]. In addition, it has been suggested that there is an "optimal narrow window" for D1-like receptor stimulation in the PFC [10,291,358] suggesting both hyper- and hypo-dopaminergic transmission in the PFC result in impaired cognitive function. Although an underlying mechanism for the cognitive impairment induced by hyper-and hypo-dopaminergic function in the PFC has not yet been determined, a concentration-dependent modulatory influence of DA on excitatory inputs reaching to the brain region was speculated as a possible neural mechanism [308].

Chronic cocaine intake has been shown to result in morphological alteration in dendrites of PFC pyramidal cells in rodents [260], decreased PFC metabolic activity [316,317] and decreased D2-like receptor availability [316] in cocaine abusers. Interestingly, the PFC brain region also showed increased metabolic activity during cue-induced cocaine craving in cocaine abusers [190]. The results from the present study showed that protracted conditioning enhanced mPFC DA response to cocaine-paired stimuli. Similarly, others found that exposure of cocaine-paired stimuli in chronically conditioned animals caused neuronal activation in the PFC [65,214]. Will these dopaminergic and morphological changes induced by chronic cocaine result in impaired cognitive function and inability to re-wire stimulus-response association? If so, will the altered cognitive function result in impulsive and compulsive cocaine-taking behavior? By answering

these questions, we can further nourish our knowledge about cocaine-addiction and thereby contribute prevention and treatment of cocaine-addiction.

CHAPTER 5. OVERALL IMPLICATIONS

Overall results from the dissertation experiments showed that responsiveness of both NAcc and mPFC DA to cocaine-paired stimuli were dependent on a particular stage of associative learning. NAcc DA levels were influenced by cocaine-paired stimuli during early phases of conditioning training while mPFC DA levels were influenced by cocaine-paired stimuli during late phases of conditioning training. These results implicate a heterogeneous response pattern of DA in the two brain regions. The results are in accordance with other findings in literature that utilized conventional rewards as a reinforcer [12], but novel in those, which utilized cocaine as a reinforcer.

Why does DA in the two brain regions respond distinctively in a different stage of associative learning? Unfortunately, there is no apparent answer available yet. However, many anatomical, physiological and pharmacological differences between dopaminergic systems of the NAcc and mPFC that have been reported ([308] review) may have resulted in the heterogeneous response pattern demonstrated in this dissertation. For example, DA neurons projecting to the NAcc and mPFC are reported to originate in anatomically distinctive areas within the VTA [302]. The mPFC has been reported to have a lower density of DAT as compared to the NAcc [160]. In addition to the low levels of DAT, the mPFC is reported to have a higher capacity to release DA along with a slower rate of clearance and metabolism as compared to the NAcc. Thereby the mPFC DA is referred to as “release-oriented” while NAcc DA is “re-uptake oriented” [58,112,114,285].

Differences in the two brain regions are also evident with intracranial self-administration of psychostimulant. For example, cocaine is reported to be self-administered directly into the mPFC [121,122], but not into the NAcc [121]. On the other hand, amphetamine has shown to be self-administered into the NAcc [144], but not into the mPFC [120]. All of these differences may have contributed to the heterogeneous response pattern of the mPFC and NAcc, yet the differences do not give an obvious answer for the question posed above.

Perhaps, the question can better be answered by rephrasing the question to "what is happening as associative learning progresses?" A neural basis of associative learning is known as "Hebbian learning" [134], which suggests that after several pairings of a strong stimulus with a weak synapse, the weak synapse is strengthened and becomes capable of triggering postsynaptic activation by itself [280]. This is referred to as synaptic plasticity, a fundamental condition for learning and memory [169]. Is it possible that remodeling of synaptic plasticity occur, as stimulus-response learning progresses and behavior become habitual, so that behavioral response can be elicited more efficiently? Findings from an electrophysiological study support the notion. It was found that task-related recruitment and firing patterns of striatal neurons changed as a result of procedural learning, suggesting reorganization of striatal plasticity has occurred as habit learning has progressed [162].

By comparing effects of lesions made in different brain areas during different stages of associative learning, Robbins and Everitt proposed that, as associative learning

transforms from Pavlovian learning to instrumental learning, the brain region mediating the goal-directed behavior is transferred from the ventral striatum, NAcc "core" (but not "shell,") to the dorsal striatum [258] where it is largely known to involve in habit learning [333]. *In vivo* microdialysis studies showed that non-contingent presentation of cocaine-paired stimuli increased DA levels in the NAcc "core" but not in the dorsal striatum while response-contingent presentation of the cocaine-paired stimuli increased DA levels in the dorsal striatum but not in the NAcc "core" [153,154].

If the differences in the response of NAcc "core" and dorsal striatum are due to reorganization of striatal plasticity, the heterogeneous response patterns of the mPFC and NAcc observed in the dissertation experiments could also be explained by the remodeling of plasticity. Perhaps plasticity with some of glutamatergic projections to the PFC and NAcc occurred across different stages of learning (Fig. 18 and 19). A brain imaging study showed that a methylphenidate challenge resulted in decreased dopaminergic response in the striatum, but increased response in the thalamus. These responses were highly correlated with cocaine-craving, in cocaine-dependent humans as compared to normal subjects [319]. Lesions of the NAcc "core" was reported to impair acquisition of Pavlovian approach behavior, while the same type of lesion after performance became habitual did not affect learned behaviors ([258] review). The NAcc has been proposed to be the neural interface between the limbic and motor system [210]. It could be that once a habitual form of performance is established, such interface may no longer be required, and remodeling of synaptic plasticity may occur for more efficient informational flow. Another possible explanation for results from the present study is the strengthening of a

projection from the mediodorsal thalamus. The mediodorsal thalamus receives sensory input, projects to the PFC, and is involved in the execution of goal-oriented behavior. It has been shown that activity changes in the PFC and the mediodorsal thalamus during a course of tone-shock avoidance conditioning training in rabbits. In the study, the PFC exhibited persistent neural activity during training whereas the mediodorsal thalamus developed the discriminative neural activity in the late stage of behavioral learning [227]. This finding suggests involvement of the PFC and mediodorsal thalamus in learning processes. Although future studies are required to test this notion, if such remodeling of plasticity occurs as learning progresses, it is not surprising to find such heterogeneous response pattern in the NAcc and mPFC during different stages of associative learning.

FIG. 18

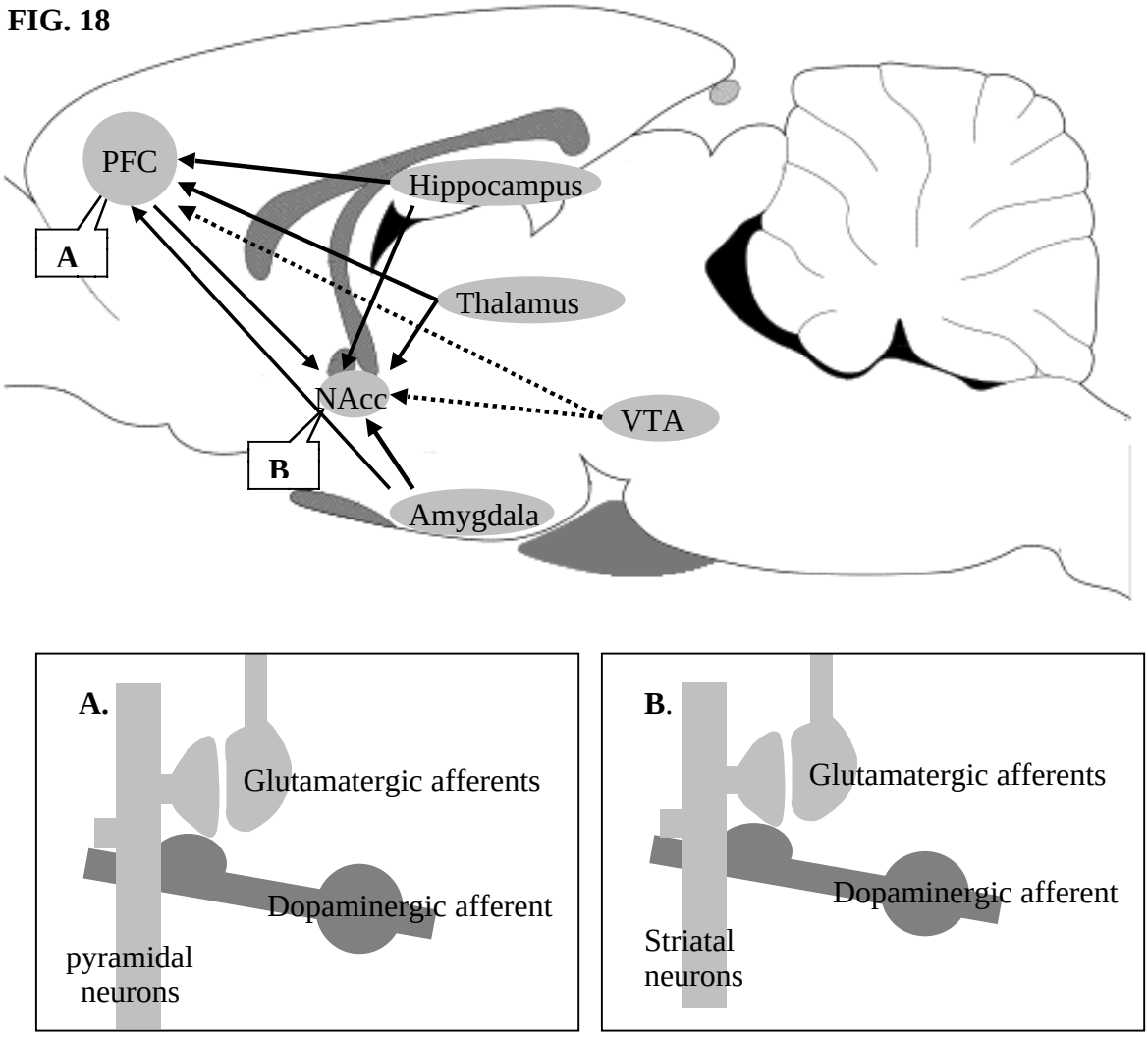

Fig. 18. NAcc and PFC receive a major dopaminergic projection from the VTA (dashed lines) [54,249]. NAcc receives glutamatergic projections (Solid lines) from the PFC, thalamus, and limbic structures such as hippocampus and amygdala [118] while PFC receives glutamatergic projections from hippocampus, amygdala, mediodorsal thalamus, contralateral mPFC [242] (and review by [131,308]), and inputs from sensory cortices [226]. The axonal varicosities of dopaminergic projection is known to make synaptic contact with a neck of dendritic spines of the pyramidal neurons in the PFC **(A)** and striatal medium spiny neurons **(B)** in close proximity to the glutamatergic synaptic inputs where it forms synaptic contact with a head of the dendritic spines of the target neurons (reviewed by [118,273]). The excitatory inputs are known to influence DA release although the exact mechanism that underlies the effect is still in debate [308]. The brain diagram was drawn with the assistance of the atlas of Paxinos and Watson (1997) [232].

Fig. 19

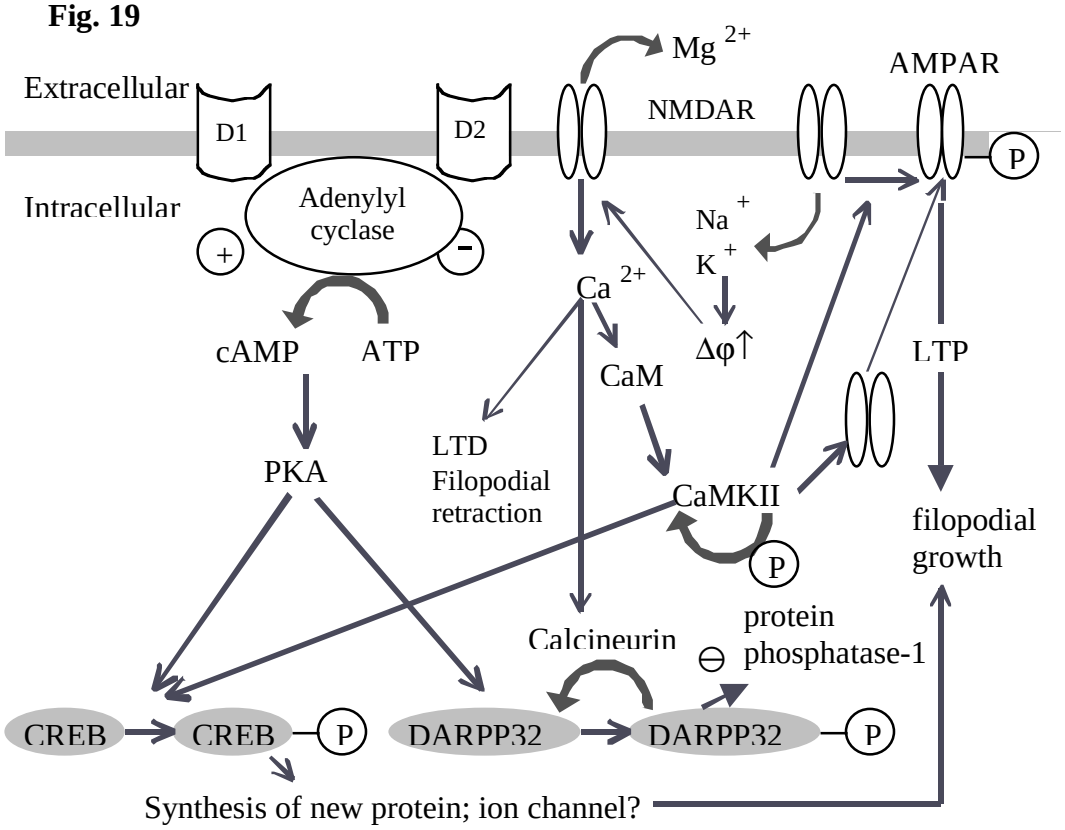

Fig. 19. Putative mechanisms for synaptic plasticity: Glutamate released from the presynaptic terminal result in influx of Na^+ and K^+ via AMPA receptors (AMPAR) that provide inward current ($\Delta\varphi\uparrow$), resulting in depolarization of postsynaptic cells. The depolarization causes release of Mg^{2+} from NMDA receptors (NMDAR) allowing Ca^{2+} to enter the cell. Ca^{2+} interact with Ca^{2+}-calmodulin (CaM), leading autophosphorylation of CaM-dependent protein kinase (CaMKII) that in turn phosphorylate GluR1 subunit of AMPAR resulting in enhancement of channel conductance or relocation of AMPAR into membrane, known as induction of LTP. AMPAR dephosphorylation or removal from synaptic membrane is, on the other hand, proposed as a mechanism of LTD (for review see [191]). The AMPAR membrane insertion is postulated to increase the size of postsynaptic density, filopodial growth, and generation of *de novo* synapses while removal of AMPAR from membrane result in filopodial retraction (for review see [189]). DA regulate phosphorylation of CREB and DARPP-32 (see detail in pp. 31). CREB and DARPP-32 are also implicated in synaptic plasticity [51,130,216]. Intracellular Ca^{2+} is known to activate CaM dependent phosphatase calcineurin, which deposhphorylate DARPP-32. Repeated administration of cocaine was shown to increase number of dendritic branches and spine density in NAcc medium spiny neurons and PFC pyramidal cells [260,261], although such morphological changes result in enhanced DA release is yet to be determined.

CHAPTER 6. THEORETICAL IMPLICATIONS OF DRUG ADDICTION AND PSYCHIATRIC PATHOPHYSIOLOGY

Jentsch suggested that chronic drug use may result in prefrontal cortical cognitive deficits such that responses to drug-related stimuli can no longer be inhibited. This results in compulsive drug-seeking and drug-taking [161]. Such a loss of inhibitory control over inappropriate behavior can also be observed in many psychiatric disorders including schizophrenia, where disrupted dopaminergic function is implicated as the pathophysiology [327].

Symptoms of schizophrenia are known to be characterized by two categories, positive and negative symptoms. Positive symptoms include thought disorders, hallucinations, and delusion while negative symptoms include flattened emotional response, inability to experience pleasure and social withdrawal [55]. Among many of the symptoms, schizophrenic patients are also known to have cognitive dysfunctions such as problems focusing attention to irrelevant stimuli, and the inability to adjust their behavior to environmental stimuli [219]. For example, schizophrenic patients are unable to discriminate between significant and insignificant stimuli while normal individuals are capable of adapting their behavior to familiar or inconsequential stimuli [250].

Abnormal functioning of the dopaminergic systems has been implicated in the etiology of schizophrenia. DA receptor antagonists have reported to alleviate symptoms of schizophrenia [57]. Paranoid psychosis induced by high dose and /or chronic cocaine

exposure, which resembles positive symptoms of schizophrenia [39,244], is also reported to be ameliorated with DA antagonists [57]. It was also found that clinical potency of antipsychotic drugs is highly correlated with affinity to D2-like receptors [69,278]. These observations suggest involvement of DA in the manifestation of symptoms of schizophrenia.

There was, however, disagreement in time course of dopaminergic agents to block DA receptors and the therapeutic effects of the drugs to appear. Neuroleptic blockade of DA receptors is achieved soon after they are administered, yet weeks of neuroleptic treatment are required before therapeutic effects are obtained [55]. Alternatively, Grace (1991) hypothesized that a low efferent glutamergic input to the striatum, caused by "hypofrontality" [152], results in a low tonic release of DA. This, in turn causes a compensatory increase in the sensitivity of postsynaptic DA receptors. The supersensitive postsynaptic DA receptors then causes episodic overstimulation of the striatal neurons in response to phasic DA release induced by environmental events, and results in manifestation of symptoms [125]. Thus, it was thought that when the mesolimbic dopaminergic system is activated by environmental events, the sensitized DA receptors over-react, and the positive symptoms of schizophrenia occur.

Grace's hypothesis was supported by the following findings. For example, "hypofrontality" was observed by brain-imaging studies that showed decreased regional blood flow [8] and glucose utilization [46] in the PFC of schizophrenic patients. A recent postmortem study also found a loss of dendrites in the PFC of schizophrenia [41]. An *in*

vivo microdialysis study offered the most compelling support for Grace's hypothesis. It was shown that the antipsychotic drug, amperozide, increased basal concentration of DA in the NAcc. This resulted in smaller behavioral responses to subsequent phasic DA release induced by amphetamine or cocaine exposure [176].

PFC damage is known to result in "stimulus-bound" or "prepotent responses," when behavior is known to be largely driven by previously learned stimuli, and an inability to re-wire such learned response results in "fixated behaviors" [204]. Schizophrenics, in which "hypofrontality" is believed to be a cause of the disease, also manifest inability to adjust their behavior to environmental stimuli [219]. The similarity between the behavioral outcome of the PFC damage and schizophrenia suggest that the PFC has an important role on behavioral flexibility. It could be that the absence of proper cortical inputs to the striatum may result in inability to remodel neural plasticity that may normally occur during learning processes. This may in turn cause inability to adjust one's behavior to stimuli. The importance of synaptic plasticity in re-wiring behavior can be observed in DARPP-32 knockout mice, which are unable to form synaptic plasticity in corticostriatal neurons [51]. The DARPP-32 knockout mice have also reported to have deficits in reversal learning of discriminative operant tasks [141]. Moreover, schizophrenic patients are indeed reported to be unable to discriminate between significant and insignificant stimuli while normal individuals are capable of adapting their behavior to familiar or inconsequential stimuli [250].

Chronic cocaine intake has been shown to result in morphological alteration in dendrites of PFC pyramidal cells in rodents [260]. In human cocaine abusers, decreased PFC metabolic activity [316,317] and D2-like receptor availability [316] was observed as compared to control subjects. Contrary to the reduced basal metabolic activity, the PFC was shown to have increased metabolic activity during cue-induced cocaine craving in cocaine abusers [190]. The results from the dissertation study also showed chronic conditioning resulted in enhanced mPFC dopaminergic response to cocaine-paired stimuli. Others also found that cocaine-paired stimuli resulted in increased neuronal activity in the mPFC [65,214]. If such altered PFC function induced by chronic cocaine exposure are similar to those found in schizophrenia and patients with PFC damage, then the cocaine-induced altered PFC function may also result in persistent saliency of cocaine-paired stimuli and fixated attraction/attention to that stimuli. This may then lead to impulsive drug-taking behavior, and initiate a vicious cycle of drug-taking habits, leading to more exposure to the drug, and thus further disruption of PFC function. Interestingly, it was found that cocaine-dependent individuals with schizophrenia are more prone to have craving elicited by cocaine-paired stimuli than those without schizophrenia [289].

Prolonged abstinence itself does not bring a malformed brain back to normal. For example, Ciccocioppo and associates showed that increased Fos-immunoreactivity induced by cocaine-paired stimuli was persistent even after 4 month of abstinence [65]. Reduced frontal activity in cocaine abusers was also found to be persistent even after 3-4 months of detoxification [316]. Thus, pharmacological manipulations of the PFC may be

necessary in order to restore the altered brain function back to normal and break the vicious cycle of drug-taking habit.

FUTURE DIRECTION OF RESEARCH

The dissertation experiments showed that NAcc DA levels were influenced by cocaine-paired stimuli during an early phase of training while mPFC DA levels were influenced during a late phase of training. Is there functional meaning for the finding? Can we prevent formation of association between cocaine and a stimulus by distracting the mesolimbic dopaminergic system during an early stage of cocaine addiction? Can we prevent cocaine craving induced by cocaine-paired stimuli by destruction of the mesocortical system at a late stage of cocaine addiction? It was found that lesions made in the NAcc "core" prevents animals from developing Pavlovian approach behavior to conditioned stimuli (review [258]), suggesting that the answer for the former question could be "yes." Manipulations of the mPFC seems however, more difficult. Dysfunction of the mPFC seems to result not only in inability to inhibit inappropriate behavior [330], but also impairment of memory consolidation [203,247]. Moreover, there seems to be an "optimal narrow window" for D1-like receptor stimulation in the PFC for proper cognitive function [10,291,358]. The theories of the "optimal" cognitive function and inability to inhibit inappropriate behavior arose from different lines of research. It is not clear whether the cognitive dysfunction results in impaired inhibition of inappropriate behavior or vise versa, or if they could also be independent of each other. Moreover, intra-mPFC infusion of D1-like receptor agents is shown to affect cognitive performance depending on preexisting individual differences in performance accuracy [128]. It is interesting to see how such individual differences interact with intra-mPFC D1 receptor

agonists and antagonists on cue-induced cocaine-seeking behavior. In addition to other molecular, neurochemical and morphological studies, such studies will further nourish our understanding of cocaine-addiction thereby contribute to the prevention and treatment of cocaine addiction.

CONCLUDING REMARKS

In the preparation of this dissertation, I became convinced that the role of DA is modulatory, at least in the NAcc and mPFC, and thus it does not have an absolute control over behavior. In fact, monoamines (both NE and DA) have been known to serve to modulate the function of widespread regions of the brain by regulating activity of a particular brain function rather than conveying specific information [56]. Moreover, there seem at least two different effects of DA on learning; DA can affect learning by modulating memory consolidation, via CREB and DARPP-32, and DA can also affect learning via modulating stimulus saliency or attentional processes. Such a modulatory role of DA makes it difficult to study its effect on animals behavior, and effects of pharmacological manipulation of DA was commonly resulted in discrepancies between findings. By elucidating details about what conditions make DA exert excitatory effects over inhibitory effects in the different brain regions, it would be very helpful for understanding the actions of drugs on behavior.

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VITA

Aiko Ikegami was born in Tokyo, Japan on October 5, 1966, the daughter of Morihiro Tokuni and Sachiko Miyamae. In 1987, she received the Associate degree in child education from Aoyama Gakuin Women's Junior College, Tokyo, Japan. During following years she was employed as a manager in an accounting department of Rolo Tomita Inc., in Yokohama, Japan. She entered Arizona State University in Phoenix, Arizona in 1994, and received the degree of Bachelor of Science from Arizona State University in May 1997. In September 1997 she entered the Graduate School of The University of Texas at Austin, Texas.

Permanent Address: 12345 Alameda Trace Circle, apt # 333, Austin, Texas 78727

This dissertation was typed by the author.